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Tropism and Morphogenesis of *Striga* Seedlings in the Host Rhizosphere

BY

C. N. WILLIAMS[1]

(*University College, Ibadan, Nigeria*)

With Plate and three Figures in the Text

Abstract

Using an agar culture technique, studies have been made on the growth and development of *Striga asiatica* seedlings in the rhizosphere of a host plant (*Sorghum vulgare*). The host root exerts a definite positive chemotropic influence over the growth of the parasite radicle in the immediate vicinity of the host root, but not further. The host root also produces a morphogenic factor which induces striking changes in the parasite seedlings. The mechanism of tropism and the significance of the morphogenic factor in the host rhizosphere are discussed.

Introduction

STUDIES on the seed germination of the root parasite *Striga* have shown that germination depends on the presence of a host-root factor (Saunders, 1933; Kumar and Solomon, 1940; Brown and Edwards, 1944 and 1946; Vallance, 1949, 1950, 1951 *a* and *b*; Solomon, 1952; Williams, 1959*a*; Sunderland, 1960). Saunders showed that roots of *Zea mays* excreted a substance (or substances) inducing germination of *Striga asiatica* Benth. (= *S. lutea* Lour.) which could be used to germinate the seeds in cultures without the host. It is now known that a number of different plants can stimulate the germination of *Striga* seeds (Andrews, 1947). Sunderland observed synergistic effects of exudates from different host species and suggested that for optimal germination there is required a complex of substances, which are produced in varying proportions by different host species.

In addition to stimulating germination, the root of the host influences the parasite in at least two other ways: firstly, by causing chemotropic curvature of the radicle of the parasite towards the root of the host, and secondly, by inducing striking morphological changes in the parasite seedling. Saunders observed that seedlings of *S. asiatica* tended to grow in the direction of the host root, although this tendency was reduced under conditions of excess moisture. The present paper reports a quantitative study on growth of *Striga asiatica* seedlings in the host rhizosphere, using agar medium to give more stable moisture conditions. The host used was *Sorghum vulgare* (var. Lt. Mori from Nigeria) which is known from field observations to be susceptible to *Striga*. In the course of the investigation, it was found that the root of *Sorghum* produces a considerable amount of acid and a diffusable factor

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having a strong morphogenic effect on the seedlings of the parasite. The mechanism of tropism and the significance of the morphogenic factor in the rhizosphere are discussed.

Experimental

Striga seeds do not germinate readily without a period of 'moisture treatment' prior to application of the germination stimulant (Brown and Edwards, 1946; Vallance, 1950). Studies on the respiratory quotient (R.Q.) of the seeds during moisture treatment have suggested that changes in respiratory substrate take place (Vallance, 1951 *a* and *b*). In addition to this, with *Striga asiatica*, germination-inhibitors are leached out of the seeds during moisture treatment

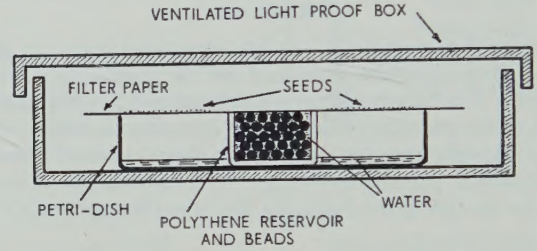

Fig. 1. Apparatus for the moisture treatment of parasite seeds.

(Williams—unpublished), together with a growth-inhibitor identifiable as the Inhibitor-β of other workers (Williams, 1959*b*).

The method of moisture treatment illustrated in Fig. 1 was found to be most convenient and gave satisfactory germination, probably because inhibitors were carried to the edge of the filter-papers by the capillary flow of water from the central reservoir. Ten days' treatment at 30–35° C. gave optimum germination.

In studying growth of the parasite seedlings in the host rhizosphere, moisture-treated seeds were mixed with still molten plain agar (1 per cent. at 40° C.–45° C.) and 10 c.c. of the mixture (containing several hundred seeds) was pipetted into 6 cm. Petri dishes. The agar was washed several times with distilled water before use to remove soluble materials which could encourage bacterial growth. One host seedling with radicle about 2 cm. long was planted in each dish just before the agar set. To reduce contamination with micro-organisms, the host seeds were surface-sterilized by shaking with bromine water for about 10 minutes. The parasite seeds could not be surface-sterilized without damage to the embryo. However, contamination by micro-organisms was not heavy, except in the region of dead parasite seeds and in old cultures. Cultures were incubated in the dark at a slight incline to the horizontal to induce the host root to grow across the dish, and the temperature was maintained at 30–35° C.

Tropism and Germination in the Host Rhizosphere

After a period of about 90 hours, curvature of the radicle of the parasite towards the host root and the departures from this tendency were scored.

Thus, if the final position of the apex of the radicle was towards the host, this was counted as being positively chemotropic. If the radicle axis was found to be parallel to the host root after careful measurement the seedling was rejected from the scoring. Tropism was scored in 4-mm. zones parallel to the host root, the zones being marked with indian-ink lines on the under sides of each dish. The combined results of three separate experiments, using 5 to 10 replicates in each, are shown in Table 1.

TABLE 1

Tropism and Germination of Striga asiatica *Seedlings at Various Distances from the Host Root*

Rhizosphere zone	Number of seedlings observed	Per cent. positive chemotropism	Per cent. germination
1. (0–4 mm.) . . .	282	76*	51
2. (4–8 mm.) . . .	196	58†	38
3. (8–12 mm.) . . .	141	55†	30
4. (12–16 mm.) . .	—	—	17
5. (16–20 mm.) . .	—	—	11

* Significantly different from random directional growth at the 1 per cent. level of probability.

† No significant difference from random directional growth.

From the table it can be seen that seeds near the host root were influenced by a substance diffusing from the host root. Variation between replicates was very high but germination always increased with proximity to the host. A significant positive chemotropism occurred in the first zone (0–4 mm. from the host root) but not in the further zones. Chemotropism was not complete however, even in the nearest zone to the host root. The reason for this is considered in the discussion below.

Morphogenic Effects of the Host Root

Striking changes in the form of the radicle of the parasite occurred in the vicinity of the host root. Growth and development in the various zones was recorded by making camera-lucida measurements at intervals. The host root seemed to induce a number of effects on the parasite including: 1, a lower rate of extension growth; 2, increased radicle diameter; 3, the production of root-hairs; 4, proliferation of cortical cells; 5, branching of the parasite radicle. The combined results of measurements of the above changes are shown in Table 2.

It is apparent that a diffusible factor from the host root brings about the morphogenic changes indicated in the table. Plate, Figs. 1 and 2 show the typical form of a parasite seedling growing in the immediate vicinity of the host root. Away from the host the parasite radicle is typically long and slender and without root-hairs. Plate, Fig. 3 shows branching of the parasite radicle which has never been observed in seedlings remote from the host root. The swelling of cortical cells (Plate, Fig. 4) occurred most frequently in the second

zone from the host root. The appearance of these seedlings indicates a breakdown of polarity in the parasite radicle.

TABLE 2

Morphogenic Effects of the Host on Striga asiatica *Seedlings at Various Distances from the Host Root*

Rhizosphere zone	No. of seedlings observed	Average no. root-hairs per seedling	Average diameter of radicle (mm.)	Swollen cortical cells (% seedlings)	Branching (% seedlings)	Average extension grown (mm.)
1. (0–4 mm.)	282	12·2	0·16	3·2	1·4*	0·45
2. (4–8 mm.)	196	2·7	0·13	7·4	0	0·95
3. (8–12 mm.)	141	1·5	0·10	4	0	1·25
4. (12–16 mm.)	66	0·86	0·11	5·5	0	—
5. (16–20 mm.)	40	0	0·10	0	0	—

* Significant since branching never occurred without the host root.

Acid Production by the Host Root

The host root produces a considerable quantity of acid which diffuses through the agar. This can be demonstrated by incorporating indicators in the agar (Plate, Fig. 5). In this experiment, indicators were added to the agar solution, itself at approximately pH 7, while still molten. The host seeds were surface-sterilized before planting in the agar and grown under aseptic conditions. It can be seen that the acidity in the region of the host plant root reached a value of about pH 4·0.

The rate of change of pH in the various zones about the host root was followed by using B.D.H. universal indicator in the medium. In this experiment, ten replicates were used, the average values being recorded in Fig. 2. In the first zone (0–4 mm. from the host root) the pH dropped rapidly, approaching a value of pH 4 within 30 hours. In zone 2 (4–8 mm.) the fall in pH was less rapid. In zones 3, 4, and 5, the acid front (indicated by a drop in pH below 7·0) appeared in the succeeding zones about 15, 25, and 35 hours from the start of the experiment and the rate of fall of pH was progressively reduced. Extrapolation of growth/time curves shows that the germination front travels much more rapidly than the acid front. The onset of germination in zones 1, 2, and 3 was approximately 12, 14, and 16 hours respectively). Consequently, acid production could be a factor in producing the morphological changes recorded in Table 2, since they are absent from the outermost zones which the acid front reaches only after the growth of the parasite has practically ceased.

Interaction of pH and a Morphogenic Factor

To investigate further the role of pH in the morphogenesis of *Striga*, the following experiments were carried out:

1. *Striga* seedlings were grown in a solution (prepared by growing about 100 seeds of *Sorghum* in sand for 10 days and eluting the root solution with 100 c.c. of distilled water) which was buffered with M/100 phosphate buffers

(KH_2PO_4/K_2HPO_4) at levels ranging from pH 5 to pH 8. This solution is termed 'dilute root solution'.

2. Seedlings were washed by decantation with distilled water after germination with the 'dilute root solution' and grown in M/100 phosphate buffers made up with distilled water.

3. A concentrate of the 'dilute root solution' used in 1 above was prepared by extracting with activated charcoal (1 g. per litre) and eluting the absorbed substances three times with 10 ml. of 95 per cent. acetone. The acetone was removed under vacuum at room temperature, leaving an aqueous concentrate

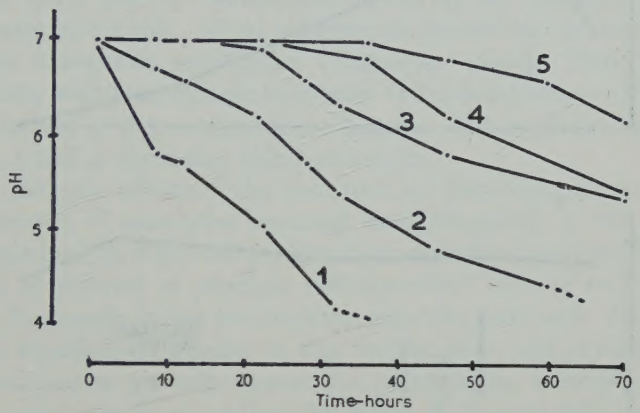

FIG. 2. pH changes at various distances from the host root—1, 0–4 mm.; 2, 4–8 mm.; 3, 8–12 mm.; 4, 12–16 mm.; 5, 16–20 mm.

having an astringent musty smell. A few drops of this concentrate were placed in wells made in agar-seed suspensions in Petri dishes as used in the previous experiments, which were buffered as above. A germination factor diffused from the agar wells and development of the parasite seedlings in a 4-mm.-wide zone about each well was recorded.

Camera lucida was used to record the development of seedlings in the above experiments. Each treatment was in duplicate, the results of both growth and root-hair formation being shown in Fig. 3.

In the 'dilute root solution', root-hair formation was significantly increased at low pH values and extension growth was reduced. This would seem to indicate that the morphological effect is due to acid diffusion from the root of the host. In distilled water, however, variation in pH between pH 5 and pH 8 had no significant effect on root-hair formation or extension growth. This seems to indicate that it is not a simple effect of acid but that a second factor is responsible for the morphogenic changes, which is more effective at low pH values. With the charcoal concentrate of the host-root solution, root-hair formation occurred even at pH 8, but was greater at lower pH values, while reduced extension growth occurred at all levels of pH but was less marked at higher values. Acid production thus appears to be not primarily

responsible for the morphogenic effects but increases the action of a morphogenic factor in the host-root solution.

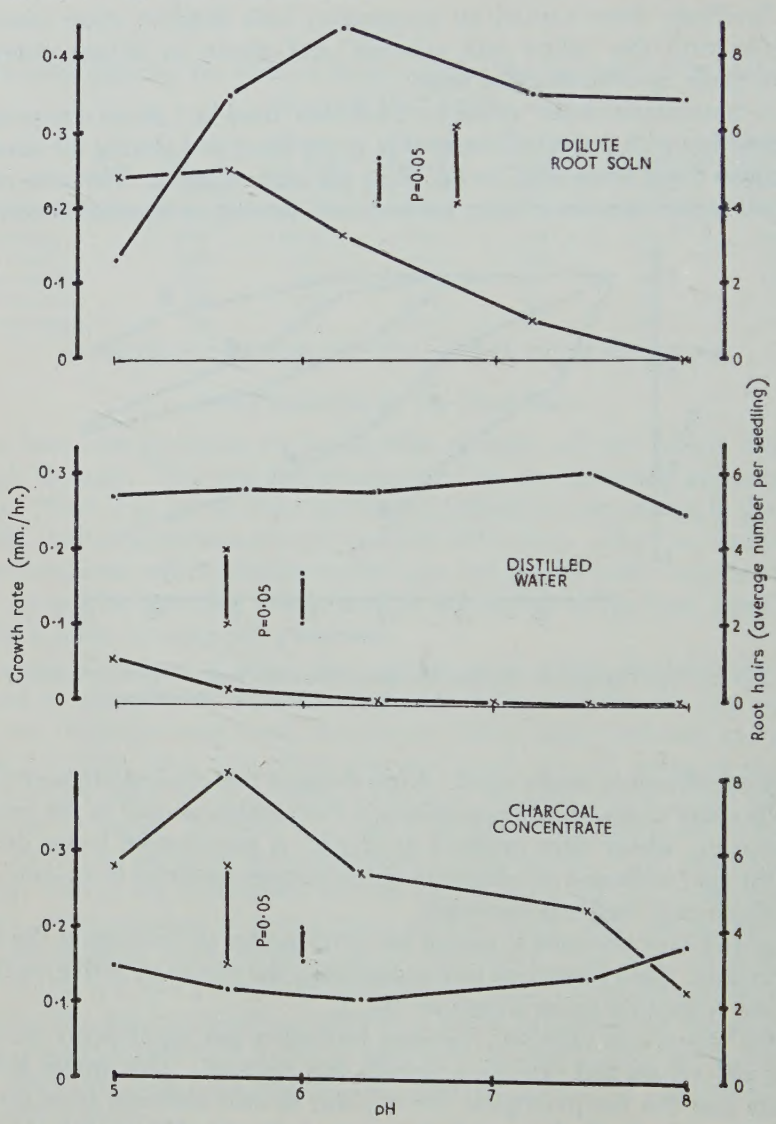

FIG. 3. Interaction of pH with root exudates and distilled water in extension growth (•—•), and root-hair formation (×—×), in *Striga* seedlings in root exudates or distilled water of varying pH.

DISCUSSION

Positive chemotropic curvature will occur if the chemotropic factor brings about an inhibition of growth on the nearer side of the responding organ. This appears to be what happens with *Striga* seedlings growing in the host

rhizosphere. Since inhibition of extension growth occurs in the region of the host root it seems likely that the same inhibitory factors are responsible for the curvature of the radicles in the direction of the host root. It is, however, true that positive chemotropism is by no means complete, even in a zone immediately adjacent to the host root. Failure to undergo positive chemotropic curvature may be partly explained by variations in orientation of the seedlings in relation to the host root. If the radicle begins its growth in a direction away from the host root, curvature could easily fail to take place. Another reason for the failure of obvious chemotropism is undoubtedly the roughly spiral growth movements shown by the parasite radicle unrelated to external stimuli. These endogenous growth movements have been described in a separate publication (Williams, 1960*a*). They take place simultaneously with chemotropic curvature and directional growth is a result of the interaction of the two curvature factors. The seedlings illustrated in Plate, Figs. 3 and 4, show this spiral curvature.

The morphogenic effects of the host root are interesting for a number of reasons. Growth of root-hairs occurred most frequently in the immediate vicinity of the host root but the proliferation of swollen cortical cells and the break-down of polarity to produce a disorganized mass of cells tended to occur most frequently in the second zone from the host root. As extension-growth was significantly greater in this second zone and proliferation was generally confined to the cells nearest the radicle apex, it would seem likely that proliferation may be a result of the action of the root-hair factor on the older seedlings in this zone from the host root, possibly because of reduced polarity in older seedlings.

It is interesting that similar effects on the development of the parasite seedlings can be induced by treatment with light, and with kinetin [6-(2 furfuryl) aminopurine] which is known to simulate the effects of light under certain conditions. The most effective wave-length in inducing these formative effects is in the blue region of the spectrum, and to a lesser extent in the red (Williams, 1960*b*). Kinetin at a level of 1 p.p.m. induced branching of the parasite radicle, very similar to that which occurred in the host rhizosphere. At higher levels (10 and 100 p.p.m.) this substance induced shoot formation. Shoot development has not, however, been observed to result from exudates in the host rhizosphere, although this does take place shortly after contact is made with the host root. Worsham *et al.* (1959) have shown that kinetin and certain other 6-substituted amino-purines can stimulate the germination of *Striga* seeds in cultures without the host, so that the possibility exists that some factor similar to kinetin may be a constituent of the germination complex. If this is so, then the concentration required for germination must be considerably lower than that required for the induction of morphogenic effects. It would seem likely that the morphogenic factor acts in some way through auxin metabolism, and this is supported by the increased activity at lower pH values (Simon and Beevers, 1952). It is interesting that the action of kinetin in root-hair formation is also increased at low pH levels. The

results of an experiment on the interaction of pH and kinetin in root-hair formation in *Striga* seedlings are shown in Table 3. It would seem likely that the action of kinetin in root-hair formation is through the same metabolic system.

TABLE 3

Interaction of pH and kinetin in root-hair formation

(Av. number of root-hairs per seedling)

	Kinetin 0	Kinetin 0·2 p.p.m.	Kinetin 1 p.p.m.
pH 5·2	0	5·0	8·5
pH 6·4	0	5·6	3·7
pH 7·4	0	1·1*	0·3*

* Significantly lower at the 2 per cent. level.

The natural germination stimulant for *Striga* seeds does not appear to have been identified, although this, and the germination stimulant for *Orobanche* seed, have been partly characterized by Brown and co-workers (1949, 1951 *a* and *b*, and 1952 *a* and *b*). The *Orobanche* stimulant appears to have a low nitrogen value indicating that it is not a purine compound. These workers have suggested that the *Striga* and *Orobanche* germination factors may be similar. More recent work by Sunderland (1960) indicates, however, that a complex of factors is probably necessary for optimal germination of parasite seeds, and these factors are produced in different proportions by different host species.

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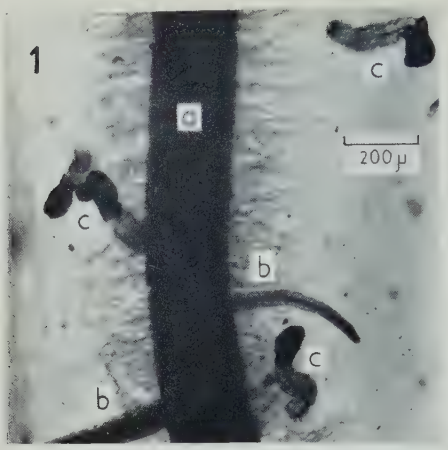
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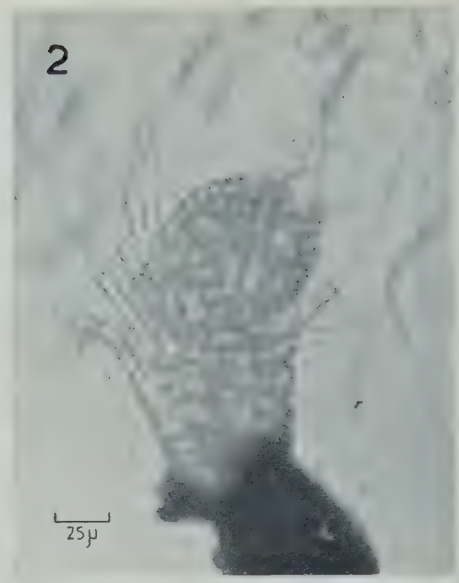
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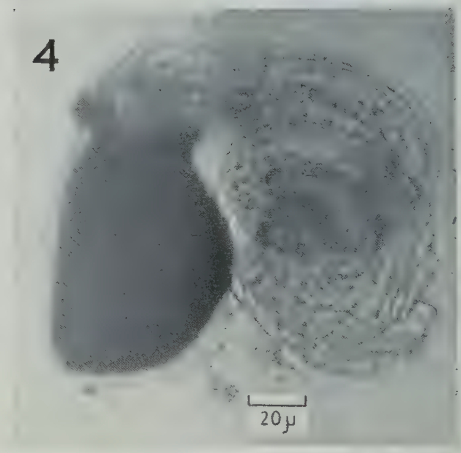
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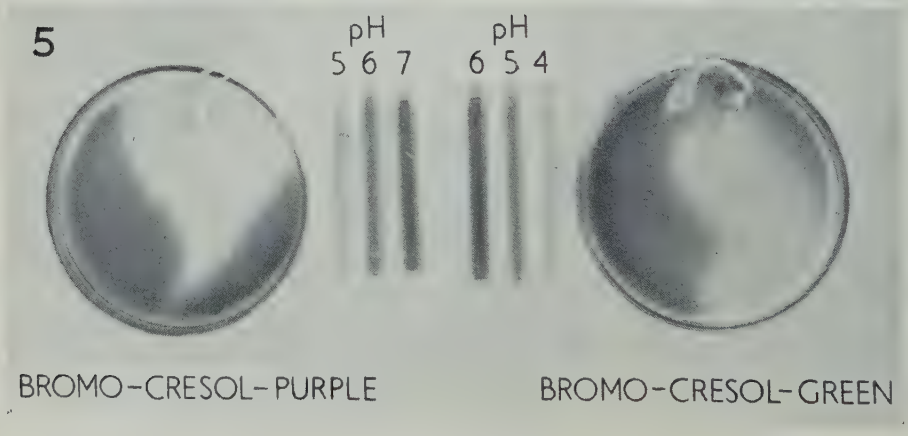
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Simon, E. W., and Beevers, H., 1952: The Effect of pH on the Biological Activities of Weak Acids and Bases. New Phytol., **51**, 163.
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EXPLANATION OF PLATE

1. Growth of *Striga* seedlings in the host rhizosphere (*a*—primary root of host, *b*—branch root, *c*—parasite seedlings).
2. Typical appearance of seedling in the host rhizosphere.
3. Branching induced in the host rhizosphere.
4. Proliferation of cells and loss of polarity induced in the host rhizosphere.
5. Acid production by the host plant root.

Types of Rot, Rate of Rotting, and Analysis of Pectic Substances in Apples Rotted by Fungi

BY

M. COLE[1] AND R. K. S. WOOD

(*Department of Botany and Plant Technology, Imperial College, London, S.W. 7*)

With one Figure in the Text

Abstract

The rate at which the fungi grew through apples was determined in various ways and used to estimate their rate of linear advance. Five fungi were studied: they were *Sclerotinia fructigena* (firm, brown coloured rot, rapid growth through apples), *Botrytis cinerea* (soft, light-brown coloured rot, rapid growth through apples), *Pyrenochaeta furfuracea* (firm to soft rot, variable in colour but generally dark, slow growth through apples), *Penicillium expansum* A (soft, white rot, slow growth through apples) and *Penicillium expansum* B (soft, white rot, medium rate of growth through apples). *S. fructigena* had the highest rate of linear advance which was about three times that of *P. furfuracea* which had the lowest.

Methods for extracting different types of pectic substances from sound and rotted tissues are described, and details are given of a rapid and reasonably accurate colorimetric method of determining the anhydrogalacturonic acid content of these extracts. The firm-rot fungi reduced the water-insoluble pectic substances by 10–20 per cent., but the soft-rot fungi caused much larger changes, up to 70 per cent. being degraded. The firm-rot and soft-rot fungi had different effects on the pectic substances insoluble in dilute acid but soluble in dilute alkali. The soft-rot fungi had little effect on these substances, or reduced their concentration, whereas the firm-rot fungi caused substantial increases compared with sound tissue. These results are considered in terms of pectic enzyme activity. Analysis of extracts by paper chromatography showed that galacturonic acid, absent from sound tissue, was present in each type of rotted tissue. Di- and tri-galacturonic acids were present in rots caused by *P. expansum*, and these rots probably also contained products from the break-down of other polysaccharides.

Introduction

FOR a variety of reasons, apples are particularly suitable for studying the mechanism by which fungi become established in plants and produce symptoms of disease. A single stock of fruit, obtained at the end of the growing season, if stored properly, may be used for most of the succeeding year. Each apple contains a large amount of relatively homogeneous parenchymatous tissue which is easy to inoculate and from which it is not difficult to extract enzymes, cell-wall constituents, and other substances. Furthermore, the tissue is quite acid so that there is little risk of bacterial contamination during the development of the rot. Another advantage arises from the fact that the

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enzymes which degrade pectic substances are important in the rotting process, and that the activity of these enzymes during the process may be assessed by comparing the quantities and types of pectic substances in sound and rotted tissue. Apples are very suitable for this purpose because unless they are overripe they contain relatively large quantities of pectic materials. Also, comparative studies are possible because there are a number of fungi which rot apples at different rates to produce rots which are quite different in appearance and texture.

A preliminary study of the rotting of apples by fungi in terms of pectic enzyme activity was made by Cole (1956), and the work reported now and in the second paper continues and extends this line of investigation. Described below are the different types of rot, the rate at which different fungi rot apples, and the extraction and estimation of the pectic substances in sound and rotted apple tissue. The second paper deals with the pectic enzymes and polyphenols in rotted tissues, with the interactions between these two groups of substances, and with their significance in the development of rots.

Methods

Fungi. These were isolated from diseased fruit, and cultures were established from single spores or hyphal tips. After pathogenicity to apples had been confirmed, stock cultures were stored at 4° under mineral oil. The fungi were: *Sclerotinia fructigena* Aderh. and Ruhl., *Botrytis cinerea* Pers., *Pyrenochaeta furfuracea* (Fr. Rostr.), *Penicillium expansum* (Link) Thom. (Strains A and B).

Apples. For almost all the work, Bramley's Seedling apples of medium size were used. These were very kindly supplied by the East Malling Research Station through the courtesy of the Director, Dr. F. R. Tubbs, and Dr. H. B. S. Montgomery. They were obtained from gas store and kept for a few days in the laboratory before they were used. Only sound, unblemished apples were used.

Pectic substances. Pectin, derived from apples, was supplied by Union Crystallex Ltd., London. It was washed in 60 per cent. v./v. ethanol containing 0·1 N-HCl, then in 95 per cent. v./v. ethanol until free of chloride, allowed to dry at laboratory temperature, and then stored in an air-tight bottle. The final product was light brown in colour and had a moisture content of about 15 per cent. w./w. The dried product contained 81 per cent. w./w. anhydrogalacturonic acid in which 74 per cent. of the acid groups were esterified.

Media. An apple-extract agar was usually used for growing fungi; 200 g. peeled apples were cooked in tap water, the suspension was passed through several layers of muslin, the pH of the extract was increased to 5·0 with N-NaOH and 20 g. agar added before adjusting the final volume to 1 l. with tap water.

Inoculation of apples. Fungi were grown on apple-extract agar in Petri dishes until the colonies were 3–5 cm. in diameter. The surface of sound fruit

was washed with water, and with 95 per cent. v./v. ethanol, and then allowed to dry. A plug of tissue, 7 mm. deep and 7 mm. diameter, was removed aseptically from the equator of the fruit and into the cavity was placed a disc, 7 mm. in diameter, taken from the edge of the fungal colony. The plug of tissue was replaced and the wound was sealed with a 1:1 mixture of petroleum jelly and paraffin wax. The apples were incubated in metal boxes at 23–25°.

Paper chromatography. Whatman no. 1 papers were irrigated with the upper layer of an *n*-butanol–acetic acid–water (4:1:5 by volume) mixture and afterwards dried at room temperature. Spots were then revealed by using the $AgNO_3$ method of Trevelyan, Procter, and Harrison (1951); the papers were left for about 10 minutes after spraying with the ethanolic NaOH in order to reveal spots with low R_f values. Sugars and related compounds appeared as brown spots on a white background. Two samples of galacturonic acid from different sources generally produced double spots under these conditions, although when this compound was produced by enzyme action a single spot was normally obtained. As little as 3 μg. galacturonic acid was detectable.

Results

General Characteristics of the Rots

Sclerotinia fructigena Aderh. and Ruhl. Five days after inoculation the rot was large, firm, brown in colour with a clearly defined front, which extended more in the eye–stalk direction than at right angles. The pustules of the fungus which appeared on the surface were sterile, but spores were produced on them after exposure to light. The skin was not easily peeled from the flesh which was as firm as sound tissue and uniformly brown in colour except for the vascular bundles which, generally, were darker. Rotted tissue became darker after exposure to air. Apples were completely rotted 10 days after inoculation; as far as could be seen, the hyphae of the fungus were always intercellular.

Botrytis cinerea Pers. Five days after inoculation a large rot was formed, free from hyphae externally, lightish brown in colour, and somewhat irregular in outline. Internally, the rot was light brown in colour with no browning of the vascular strands, it was quite soft and it was easily separated from the skin and sound tissue. Apples became completely invaded in about 19 days and the hyphae were intercellular.

Pyrenochaeta furfuracea (Fr.) Rostr. This is the conidial stage of *Phacidiella discolor* (Mount. and Sacc.) Potebnia. It was isolated from fruit imported from Australia and has, apparently, not been recorded previously for the United Kingdom. The original fruit (variety Sturmer) was almost completely invaded, with the surface black and covered with small swellings which were probably pycnidia. This condition could not be reproduced exactly in Bramley's Seedling fruit. Seven days after inoculation a small rot was produced; externally, it was dark brown in colour with no hyphae visible, and was circular with a sharply defined front. Internally, the rot had an irregular front, was fairly firm, and was brown in colour. The skin was easily peeled

from rotted tissue. Apples were completely rotted in 40 days and exposed rotted tissue smelt of alcohol, was quite soft, and was variously coloured from purple to yellow-brown or dark brown. The hyphae were intercellular. A few fertile pycnidia appeared on fruit kept for about 8 months after inoculation.

Penicillium expansum Link (Thom.) (Strains A and B). Both fungi produced similar rots, but strain B was more active. Circular rots with sharp outlines were produced after 7 days; the surface was light brown in colour and free from hyphae. Rotted tissue was very soft and watery, and white or very light brown in colour except for the vascular strands which were dark brown. Hyphae were intercellular and rots were easily separated from sound tissue and from the skin. Apples became completely invaded in about 30 days and covered with heavily sporing conidial tufts. Cavities made in the rots became lined with coremia of Strain A and sessile aggregates of conidiophores of Strain B.

Rate of Invasion of Apples

Sets of apples were inoculated with each of the fungi or left uninoculated. The apples were inoculated mid-way between eye and stalk, at two opposite points with *S. fructigena* which invaded fruit most rapidly, and equidistantly at three points with the other fungi. Immediately after inoculation the apples were weighed to within 0·1 g., and their volume determined by displacement of water. Five days after inoculation, samples of fruit were taken from each treatment and the dimensions of each rot along the surface in the eye-stalk direction and at right angles were measured. The volume and weight of each apple were measured again, the rotted tissue removed with a rubber spatula, and the volume and weight of the apple redetermined. Samples of the rotted tissue were dried at 70° to determine the dry-matter content, and the rest of the tissue was stored at −18°. Uninoculated apples were treated in the same way.

The process was repeated with another sample 7 days after inoculation, and the remaining apples were kept until they were completely rotted.

The data obtained for the weights and volumes of rots are shown in Table 1.

The volumes and weights of rots caused by the different fungi corresponded closely, so that although the rots differed considerably in appearance and texture each fungus must have caused about the same loss in fresh weight per unit volume rotted. It will be seen later that similar results were obtained on a dry-weight basis.

The fungi which had been selected for this work rotted the fruit at different rates. If the rate of rotting (by volume) by the least active fungus *P. furfuracea* is considered to be 1, the rates for the other fungi were, approximately, *P. expansum* A 1·3, *P. expansum* B 2·3, *B. cinerea* 3·6, and *S. fructigena* 7·7. The most active fungus, *S. fructigena*, had rotted about a third of an apple in 5 days.

If the last four columns of the table are considered, the rate of rotting as

given by weight or volume of rotted tissue is not the same when measured over 5 days as it is when measured over 7 days, being greater for *S. fructigena*, and less for the other fungi. This would be expected if the linear rate of advance of the fungus through the tissue is constant. Gregory and Horne (1928), assuming that apples are spherical, derived the relation

$$V = (y/R)^3(8-3y/R)/16$$

between V the volume of the rot as a proportion of the volume of the apple, R the radius of the apple, and y the radius of the rot which is assumed to start at the surface.

TABLE 1

Weights and Volumes of Rots in Apples

	Wt. (g.) rot per inoculum		$\frac{\text{Wt. rot}}{\text{Wt. apple}} \times 100$		g. rot/hr.		% apple rotted/hr.	
	in 5 days	in 7 days	in 5 days	in 7 days	over 5 days	over 7 days	over 5 days	over 7 days
S. fructigena	49·1±4·5*	55·9±8·0	35·7	45·0	0·41	0·33	0·30	0·27
B. cinerea	20·8±7·8	31·1±3·2	15·7	25·2	0·16	0·18	0·12	0·14
P. furfuracea	4·1±1·8	8·8±3·3	3·1	6·4	0·03	0·05	0·03	0·04
P. expansum A	6·1±0·5	12·0±3·0	4·3	8·7	0·05	0·07	0·03	0·05
P. expansum B	11·0±1·1	21·7±2·5	8·4	16·3	0·09	0·13	0·07	0·09

	Vol. (ml.) rot per inoculum		$\frac{\text{Vol. rot}}{\text{Vol. apple}} \times 100$		ml. rot/hr.		% apple rotted/hr.	
	in 5 days	in 7 days	in 5 days	in 7 days	over 5 days	over 7 days	over 5 days	over 7 days
S. fructigena	56·0±5·3	64·4±9·5	34·5	44·0	0·47	0·38	0·29	0·26
B. cinerea	23·6±8·6	36·7±4·8	15·2	25·4	0·19	0·21	0·12	0·15
P. furfuracea	5·3±3·2	11·6±3·3	3·3	7·0	0·04	0·07	0·03	0·04
P. expansum A	8·1±0·8	14·3±3·4	4·9	8·9	0·06	0·08	0·04	0·05
P. expansum B	13·3±1·8	24·8±3·7	8·6	15·9	0·11	0·14	0·07	0·09

* Limits of error at $P = 0·05$

This formula was used with some of the data given in Table 1 to calculate the rate of advance of the front of the rot (y/time). The results are shown in Table 2.

These values are somewhat high because no allowance has been made for the fact that the rots started not at the surface but 7 mm. below. Except for *S. fructigena* the rate of linear advance of the fungus over the first 5 days was close to that over 7 days, although in each case it was slightly higher. This difference was considerably greater with *S. fructigena* which advanced more rapidly. It will also be seen that whereas after 5 days *S. fructigena* had produced about 12 times as much rot as had *P. furfuracea*, the rate of linear advance of *S. fructigena* was only about 2·5 times that of *P. furfuracea*.

The rate of linear advance was also calculated from the external dimensions of the rot along the surface in the eye–stalk direction and at right angles to this. The relation between the radius of the rot and these dimensions was determined empirically (from a scale drawing) for rots of different radius on the assumption that the rot originated 7 mm. below the surface. The radius

of the rot was then estimated from known values of the external dimensions the results are shown in Table 3.

There is, in general, good agreement between the results of Tables 2 and 3. The rates of linear advance estimated after 5 and 7 days agree remarkably well, so that it would seem that once it is established that the rot spreads

TABLE 2

Rate of Linear Advance of Rot

	Linear advance mm./hr.	
	over 5 days	over 7 days
S. fructigena	0·30	0·24
B. cinerea	0·20	0·17
P. furfuracea	0·12	0·11
P. expansum A	0·13	0·12
P. expansum B	0·16	0·15

TABLE 3

Rate of Linear Advance of Rot Estimated from Measurements of External Dimensions

	Linear advance mm./hr.	
	over 5 days	over 7 days
S. fructigena	0·30	0·29
B. cinerea	0·17	0·17
P. furfuracea	0·10	0·10
P. expansum A	0·12	0·11
P. expansum B	0·14	0·13

spherically from the point of origin, the rate of advance of the fungus through the tissue can be reliably estimated from measurements of the external dimensions of the rot. Clearly this might be used to eliminate sampling in experiments designed to study the effects of different conditions on the movement of fungi through apples.

Dry-weight Changes

These are shown in Table 4 and are the means for three determinations.

After 5 days and 7 days rotted tissue contained slightly less dry matter than sound tissue, with indications that *P. furfuracea* reduced the dry weight more than did the other fungi. As might be expected, the percentage dry weight of the tissue when the apple was completely rotted was inversely related to the time taken for the rot to occupy the whole of the fruit.

Pectic Substances in Sound and Rotted Tissue

When pectic enzymes of host and pathogen play a part in the development of rots, their activity during this process should be reflected in qualitative and quantitative changes in the pectic substances originally present in the sound tissue. Analyses were therefore made of these substances present in healthy apples and in tissue which had been rotted by each of the five fungi.

Estimation of Galacturonic Acid and Pectic Substances

Cole (1956) used the Carré and Haynes (1922) calcium pectate method in a similar investigation, but this method was considered to be too tedious for the many analyses which it was intended to make. The colorimetric method of Dietz and Rouse (1953) seemed the most suitable of others which have been described, and after it had been tested and somewhat modified it was the one finally selected. It is based on the specific colour reaction given by hexuronic acids with carbazole and described by Dische (1947).

TABLE 4

Dry-weight Changes in Sound and Rotted Tissue

	% Dry weight			
	After 5 days	After 7 days	When completely rotted	Days to complete rot
S. fructigena	11·5	10·8	11·7	10
B. cinerea	11·1	10·9	11·4	19
P. furfuracea	10·5	10·6	6·5	40
P. expansum A	11·6	10·9	7·1	37
P. expansum B	11·6	11·3	9·5	28
Uninoculated tissue	11·8	12·0	10·6*	—

* Sound apples after 30 days.

Dietz and Rouse (1953) recommend that 0·5 ml. of a 0·1 per cent. w./v. solution of carbazole in ethanol be added to 1 ml. of the test solution; 6 ml. pure H_2SO_4 are then added, the mixture shaken, and the colour density measured 10 minutes later at 525 mμ against a blank containing each of the above substances except carbazole. After many tests it became clear that the most critical part of the assay was the adding of the H_2SO_4; the colour which developed depended upon the rate at which it was added and the rate of heat loss subsequently. Both of these were, therefore, standardized carefully. Six ml. of the acid were run from a burette into a special vessel from which a capillary led into the lagged reaction tube containing a mixture of 1 ml. of the aqueous test solution and 0·5 ml. 0·1 per cent. w./v. carbazole in ethanol; at laboratory temperatures, the acid passed through the capillary in 100 sec. Three minutes after starting to add the acid the reaction tube was stoppered and shaken to mix the contents, it was then left for a further 7 minutes before the optical density of the contents was measured in an EEL colorimeter with a no. 624 filter (525 mμ). The blank, containing 1 ml. water instead of a solution of galacturonic acid or pectic substances, was treated in exactly the same way and the reading obtained with it was subtracted from that obtained with the test solution to give a final corrected value. The same acid vessel was used in all subsequent tests.

Solutions of galacturonic acid and an apple pectin were used to obtain calibration curves. Pectin (100 mg.) which had been dried at 70° for 24 hours was dissolved in water, 50 ml. N-NaOH were added drop by drop with stirring

to saponify the pectin and neutralize acid groups, and the solution made up to 1 l. Dry galacturonic acid (100 mg.) was also dissolved in water, the same amount of NaOH was added, and the solution made up to 1 l.

Samples containing between 5 and 100 μg./ml. pectin or galacturonic acid were analysed by the above method with the results shown in Fig. 1. Straight-line relationships were obtained between the absorption at 525 mμ and concentration of galacturonic acid or pectin. The deflection unit on the colorimeter corresponded to 1·71 μg. galacturonic acid monohydrate (mol. wt.

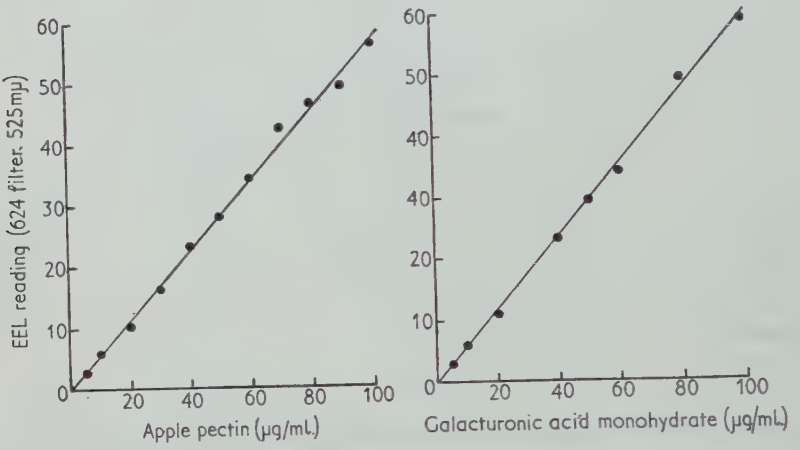

FIG. 1. Calibration curves for the colorimetric estimation of dry apple pectin and galacturonic acid monohydrate.

212) and, therefore, to 1·42 μg. anhydrous acid on the assumption that the sample was pure. For the pectin, one deflection unit corresponded to 1·74 μg. so that it can be considered to contain 1·42/1·74 = 81·6 per cent. w./w. anhydrogalacturonic acid. From the already determined saponification values of the pectin it can be calculated that the dried sample contained 86·4 per cent. w./w. pectin and that, of the available carboxyl groups, 73·8 per cent. were esterified.

Extraction of Pectic Substances from Tissue

Many different methods have been used to do this in the past, but only a recent one, that of Dietz and Rouse (1953), will be dealt with in detail because it formed the basis of the method which was used extensively later in this work. Originally, the method was used to extract pectic substances from citrus concentrates. It was now used with suspensions of apple tissue prepared in the following way. Frozen tissue (100 g.) was added piecemeal to 200 ml. ice-cold water and then blended until a fine suspension had been obtained. The volume was then increased to 500 ml. The suspension was agitated mechanically while 10 ml. samples were transferred to 50 ml. borosilicate centrifuge tubes, or to weighing bottles for dry-weight determinations. 30 ml. 95 per cent. v./v. ethanol were added to the sample in the tube which

was then placed in a water bath at 85° for 10 minutes before centrifuging. The supernatant solution was rejected, 40 ml. 60 per cent. v./v. ethanol were added, and the procedure repeated. The residue was extracted twice with 40 ml. water, twice with 40 ml. 0·4 per cent. w./v. sodium hexametaphosphate (SHMP), and twice with 40 ml. N/20 NaOH, all at 20–22° for 10 minutes. Excess (5 ml.) N-NaOH was added to each extract to saponify pectinic acids before the volume was increased to 100 ml., from which 1 ml. samples were taken for analysis by the carbazole method given above. Final results were expressed as mg. anhydrogalacturonic acid/g. dry tissue extracted.

The results obtained with the Dietz and Rouse procedure were compared with those obtained by using SHMP and NaOH at 85° instead of 20°, and by refluxing with one or more of the following solvents, N/75 HCl, N/75 NaOH, 0·5 per cent. w./v. ammonium oxalate. Also, the effect of altering the concentration of ethanol which was used in the preliminary extraction of the material was investigated.

The results, which will not be given in detail, showed the following:

(*a*) The Dietz and Rouse procedure was not satisfactory for apple tissue. When the temperature of the solvents was increased from 20 to 85°, substances dissolved by 0·4 per cent. w./v. SHMP increased by 80 per cent. and those dissolved by N/20 NaOH increased by 50 per cent.

(*b*) A sample of tissue extracted three times by refluxing with 0·5 per cent. w./v. ammonium oxalate yielded 27·6, 4·8, and 1·1 = 33·5 mg. anhydrogalacturonic acid/g. A corresponding sample refluxed twice with N/75 HCl gave 39·0 and 5·6 = 44·6 mg. and the residue refluxed twice with N/75 NaOH gave 1·8 and 0·4 = 2·2 mg. The total extracted by these treatments was, therefore, 46·8 mg. which compares with 33·5 mg. extracted by ammonium oxalate. When the residue left after refluxing with the oxalate was twice refluxed with N/75 HCl, yields of 8·6 and 2·2 = 10·8 mg. were obtained to give a grand total of 44·3 mg. which compared with the figure of 44·6 obtained with the acid alone. Clearly, therefore, ammonium oxalate cannot be regarded as a satisfactory solvent for the pectic materials in apples.

(*c*) It was found that after tissue had been extracted by refluxing with N/75 HCl and then with N/75 NaOH in the way described under (*b*), no more pectic materials were extracted when the residue was refluxed with 0·5 per cent. w./v. ammonium oxalate.

(*d*) Extraction with hot SHMP followed by hot NaOH yielded slightly more anhydrogalacturonic acid than did refluxing with ammonium oxalate which was known to extract incompletely compared with N/75 HCl and N/75 NaOH.

The Dietz and Rouse procedure is, therefore, not satisfactory for the complete extraction of water-insoluble pectic substances but becomes so if the final residue is extracted by refluxing with N/75 HCl.

(*e*) Examination by paper chromatography of the extracts of water-insoluble pectic substances in N/75 HCl, N/75 NaOH, 0·4 per cent. w./v. SHMP, and N/20 NaOH used in the above experiments showed that none contained sugar

or sugar derivatives which moved on paper chromatograms irrigated with the upper layer of an *n*-butanol-acetic acid-water (4:1:5 by volume) mixture.

(*f*) In the Dietz and Rouse procedure, after the preliminary extraction with 76 per cent. v./v. ethanol, the residue is extracted again with 40 ml. 60 per cent. ethanol to complete the removal of low molecular-weight sugars and sugar derivatives, while at the same time leaving behind the higher molecular-weight pectic substances some of which would be soluble in water. In a typical experiment the yield of water-soluble pectic substances after this procedure was 14·6 mg./g. When, however, a similar sample was extracted a second time with 60 per cent. v./v. ethanol the yield of water-soluble pectic materials fell to 5·4 mg./g. This showed that the preliminary extractions with ethanol were critical, so the solubility of pectin in different concentrations of ethanol, and the effect of using different concentrations of ethanol in the preliminary extractions were investigated. The highest yields of water-soluble pectic materials were obtained when 40 ml. 95 per cent. v./v. ethanol were added to the 10 ml. sample of tissue, the mixture kept at 85° for 15 minutes, cooled in iced water, centrifuged, the supernatant discarded, and the procedure repeated with the residue. The hot ethanol had the advantage of removing air from the precipitate so that a firm pellet was formed on centrifuging. Galacturonic acid added to a sample of the suspension was removed by this procedure, which was the one adopted in most of the subsequent work.

Method for the Extraction of Pectic Substances from Apples

On the basis of the results summarized above, the following procedure was adopted for the extraction of different types of pectic substances from sound and rotted apple tissue.

1. The skin, agar plug, and wax were removed from rotted apples and the whole of the rotted tissue transferred to clean jars which were then closed with air-tight lids, cooled as quickly as possible, and frozen at −18°. Large rots were sliced before they were placed in the jars. Sound apples, incubated for the same time as inoculated apples, were peeled, cored, and the remaining tissue sliced into jars which were then cooled and frozen. Tests showed that there were no readily detectable changes in pectic substances over several months when tissue was stored at −18°.

2. For analysis the frozen tissue was broken up into small fragments, mixed, and 100 g. weighed to within 0·5 g. while the tissue was still frozen. This sample was then added piecemeal to 200 ml. water at 0° in a blender and macerated; a further 100 ml. water were added to the mixture which was macerated for 2 to 4 minutes to give a fine suspension. The air in the suspension was removed under vacuum, and the volume increased to 500 ml. Samples (10 ml.) were removed from this suspension while it was being agitated. Three samples were used to obtain dry weights after drying at 70° for 30 hours. Others were placed in 50 ml. borosilicate centrifuge tubes to which 40 ml. 95 per cent. v./v. ethanol were added immediately. The tubes were placed in a water bath at 85° for 10 minutes, centrifuged, the supernatant

discarded, and the residue extracted with another 40 ml. 95 per cent. v./v. ethanol at 85° for 10 minutes.

3. 40 ml. water at 20–22° were added to the residue, which was stirred intermittently for 15 minutes before centrifuging for 10 minutes. The supernatant was poured into a 100-ml. volumetric flask through a plug of glass wool which caught some of the particles which became resuspended. The glass-wool plug was returned to the tube containing the residue and the extraction repeated with another 40 ml. water. Four samples, *a*, *b*, *c*, and *d*, were treated in this way.

4. The residues (including those caught on the glass wool) of samples *a* and *b* were extracted in the way described under (3) first with two lots of 40 ml. 0·5 per cent. w./v. SHMP at 85°, and then with two lots of 40 ml. N/20 NaOH at 85°.

5. The residues and glass-wool plugs from (4) were transferred to a 250 ml. reflux flask with 40 ml. N/75 HCl and refluxed for 1 hour before centrifuging and then filtering through glass wool into a 100 ml. flask. The refluxing was repeated with a second 40 ml. of N/75 HCl.

6. The residues of samples *c* and *d* were separately put into 250-ml. reflux flasks with 40 ml. N/75 HCl, and then treated as described in (5), except that sound tissue was extracted a third time.

7. The residue from (6) was extracted twice by refluxing with 40 ml. N/75 NaOH for 1 hour.

8. 5 ml. N-NaOH were added to each extract and the volume increased to 100 ml. The extracts were kept at 5° for no longer than 18 hours before analysis. The 100-ml. flasks containing the different extracts were brought to room temperature and two samples from each flask were analysed by the carbazole method already described. The extraction solvents instead of the extracts were used for blank determinations.

The analysis of one lot of rotted or sound tissue took one day when the water—SHMP—NaOH—HCl procedure alone was used, and about a day and a half when the water—HCl—NaOH procedure was used as well. This is considerably shorter than the time which would be required by the Carré and Haynes (1922, 1925) method.

Pectic materials in sound and rotted tissue. The results obtained with fruit which had been incubated at 22–23° for 7 days after inoculation are given in Tables 5 and 6 in which the figures shown have already been adjusted to allow for the different per cent. dry weight of sound and rotted tissues. The tissue was extracted with 76 per cent. v./v. and 95 per cent. v./v. ethanol at 85° before extracting with water and other solvents.

The results obtained by the two methods were essentially similar, so for the most part they will not be considered separately. The following are the main points which emerge.

1. Each of the fungi caused some degradation of pectic substances in the host tissue. The greatest losses were found in the soft rots produced by *B. cinerea* or *P. expansum* in which up to 48 per cent. of the pectic substances

originally present were degraded. In the firm rots caused by *S. fructigena* and *P. furfuracea* the losses were much less.

2. The figures for water-soluble pectin in Tables 5 and 6 agree well except for sound tissue. Data from another experiment suggest that the figure in Table 5 for sound tissue is too high and if it is accepted that the figure shown in Table 6 is more nearly correct, it is seen that *S. fructigena* and *B. cinerea*

TABLE 5

Pectic Substances Extracted from Sound and Rotted Apple Tissue Successively by Water—SHMP—NaOH—HCl

	Each tissue sample extracted by these solvents in the order shown				Total of the fractions not soluble in water	Total of all fractions
	1	2	3	4		
Tissue	Water	0·4% w./v. SHMP	N/20 NaOH	N/75 HCl		
Sound apple	18·4	31·5	2·4	9·6	43·5	61·9
S. fructigena rot	14·5	23·6	6·9	7·7	38·3	52·8
	(−21)	(−25)	(+188)	(−20)	(−12)	(−15)
B. cinerea rot	15·7	12·0	7·8	5·0	24·8	40·5
	(−15)	(−62)	(+224)	(−48)	(−43)	(−35)
P. furfuracea rot	13·5	26·8	9·0	10·0	45·8	59·3
	(−27)	(−15)	(+275)	(+4)	(+5)	(−4)
P. expansum A rot	23·8	10·2	7·2	6·4	23·8	47·6
	(+29)	(−68)	(+200)	(−33)	(−45)	(−23)
P. expansum B rot	19·1	7·2	2·9	4·5	14·6	33·7
	(+4)	(−77)	(+21)	(−53)	(−67)	(−46)

All figures not in brackets are mg. anhydrogalacturonic acid/g. dry weight sound tissue invaded.

Figures in brackets are the % loss or gain of pectic substances compared with sound tissue.

have little apparent effect on the level of this type of pectin, *P. furfuracea* reduces it by about 12 per cent., whereas the two isolates of *P. expansum* cause it to increase. This anomalous result will be dealt with later.

3. Each of the fungi reduced the amounts of pectic substances extractable by SHMP or HCl. The residue after extraction by SHMP still contains a fraction soluble in HCl, and the total extracted first by SHMP and then by HCl is close to that extracted by HCl alone. This suggests that the HCl fraction of the Carré and Haynes method contains at least two fractions the larger of which is soluble in SHMP and consists of low methoxyl pectinates of calcium and magnesium, whereas the smaller fraction consists of compounds which are insoluble because of their much higher molecular weight, or because they are bound to other insoluble components of the cell wall. The data in Table 5 suggest that the soft-rot fungi degrade more of this fraction than do the two firm rot fungi.

4. The figures in Tables 5 and 6 for material soluble in NaOH cannot be compared directly because the extractions with NaOH were preceded by different treatments. The data in Table 6 are likely to be the more significant

because it is known that extraction by SHMP is incomplete. They show that two of the soft-rot fungi cause little change, and that the third reduces the amount of material soluble in NaOH, whereas the two firm rot fungi cause substantial increases. These differences are explicable in terms of the activities of pectic enzymes in the rots. In the firm rots it may be supposed that there is little chain-splitting activity but considerable pectinesterase activity, so that pectic acid, insoluble in HCl, would accumulate. In contrast, although pectinesterase may be present in soft rots, the pectic acid which is produced

TABLE 6

Pectic Substances Extracted from Sound and Rotted Apple Tissue Successively by Water—HCl—NaOH

	Each tissue sample extracted by these solvents in the order shown			Total of the fractions not soluble in water	Total of all fractions
	1	2	3		
Tissue	Water	N/75 HCl	N/75 NaOH		
Sound apple	15·7	42·6	2·7	45·3	61·0
S. fructigena rot	15·0 (−4)	32·8 (−23)	6·7 (+148)	39·5 (−13)	54·5 (−11)
B. cinerea rot	15·4 (−2)	18·5 (−57)	3·6 (+33)	22·1 (−51)	37·5 (−39)
P. furfuracea rot	13·7 (−13)	38·2 (−10)	8·0 (+196)	46·2 (+2)	59·9 (−2)
P. expansum A rot	24·3 (+55)	19·0 (−55)	3·0 (+11)	22·0 (−52)	46·3 (−24)
P. expansum B rot	18·4 (+17)	11·7 (−73)	1·6 (−41)	13·3 (−71)	31·7 (−48)

All figures not in brackets are mg. anhydrogalacturonic acid/g. dry-weight sound tissue invaded.
Figures in brackets are the % loss or gain of pectic substances compared with sound tissue.

does not accumulate because the soft-rot fungi produce chain-splitting enzymes which remain active in the rotted tissue. Further evidence along these lines will be given in the second paper.

5. Comparison of the data in Tables 5 and 6 with those of Table 3 shows clearly that the rate at which the fungus spreads through the tissue is not related to the rate at which pectic materials are degraded. Thus, *P. expansum* B, which moves through apples less than half as quickly as *S. fructigena*, degrades about three times as much of the total pectic substances.

Nature of water-soluble pectic substances. The tissues from which the results shown in Tables 5 and 6 were derived were extracted first with 76 per cent. v./v. and then with 95 per cent. v./v. ethanol before they were extracted with water. In parallel experiments, the effect of replacing 95 per cent. v./v. by 60 per cent. v./v. ethanol was studied and the results are shown in Table 7.

The action of the fungi increased the concentration of pectic substances soluble in 60 per cent. ethanol but insoluble in 95 per cent. ethanol. The

increases were larger for the soft rots than for the firm rots, and were greatest for the two rots in which there was most degradation of water-insoluble pectic substances. The results show that in the rotted tissue there accumulate pectic substances of low molecular weight which are water soluble and which are derived from compounds of higher molecular weight by the action of enzymes produced by the fungi; these compounds of low molecular weight would become less soluble as the concentration of the ethanol in the solvent increased.

TABLE 7

Water-soluble Pectic Substances after Different Ethanol Extractions

Tissue	76% and 95% (v./v.) ethanol	76% and 60% (v./v.) ethanol	Difference
Sound apple	16·9*	15·6	1·3
S. fructigena rot	14·2	9·4	4·8
B. cinerea rot	15·5	5·4	10·1
P. furfuracea rot	13·6	6·0	7·6
P. expansum A rot	24·0	9·2	14·8
P. expansum B rot	18·8	4·5	12·3

* Water-soluble pectic substances expressed as mg. anhydrogalacturonic acid/g. dry-weight sound tissue invaded.

It might be considered that a better picture of the activity of the fungi in apples would be given by figures based on extractions with 76 and 60 per cent. v./v. ethanol. This would give losses in total pectic substances as follows: *S. fructigena* 20 per cent.; *B. cinerea* 55 per cent.; *P. furfuracea* 14 per cent.; *P. expansum* A 49 per cent.; *P. expansum* B 71 per cent.

Paper Chromatography of Extracts from Sound and Rotted Tissues

Juice pressed from rotted tissue 9 days after inoculation with *S. fructigena*, and 16 days after inoculation with the other fungi, and from sound tissue from apples which had been incubated for 9 and 16 days, was made cell-free by centrifuging and brought to pH 5·0 with concentrated NaOH before 0·02 ml. was applied to small areas on the paper. Typical results are given in Table 8.

Comparison with the results obtained by other workers and with results obtained after pectin had been degraded *in vitro* by pectic enzymes made it likely that the definite spot D_1 which was present in the juice from the *P. expansum* rots was di-galacturonic acid, and that spot F appearing clearly in *P. expansum* A rot juice and as a trace in two other juices, was tri-galacturonic acid. The identity of spots A, B, C, E, and G was not established.

Galacturonic acid was not present in the juice from sound tissue, but was present in the juice from each of the rots. The spot was smallest for the *S. fructigena* rot in which there was relatively little break-down of pectic substances. The prominence of the spot for the *P. furfuracea* rot was surprising, but this is probably explained by the fact that the juice was obtained from rots 16 days old which are quite soft in contrast to the firm rots produced 7 days after inoculation and which were those used in earlier analyses. If the

identification is correct, di- and tri-galacturonic acid are absent in juices from *S. fructigena* and *B. cinerea* rots, and present only in traces in *P. furfuracea* rots, whereas both are readily detectable in rots caused by *P. expansum* A, and di-galacturonic acid is obtained from *P. expansum* B rots. The absence of tri-galacturonic acid from the *P. expansum* B rot is probably related to the fact that this is the rot in which there was the greatest degradation of the pectic substances. The results also suggest that the mechanisms, by which *P. expansum* and the other fungi degrade pectic materials *in vivo*, are different; further evidence that this is so will be given in the second paper.

TABLE 8

Paper Chromatography of Juice from Sound and Rotted Apple Tissues

Spots	Sound apple	S. *fructigena* rot	B. *cinerea* rot	P. *furfuracea* rot	P. *expansum* A rot	P. *expansum* B rot	R_{GA} 1	R_{GA} 2
Fructose	++++	++++	++++	++++	++++	++++	1·53	..
Glucose	++++	++++	++++	++++	++++	++++	1·22	..
Galacturonic acid	−	+	+++	+++	++	+++	1·00	1·00
Sucrose	+++	+−	−	−	++	++	0·79	0·81
A	−	−	++	++	+	+	0·67	0·69
B	+	++	++	++	++	++	0·56	0·57
C	−	−	+	+	+−	+−	0·42	0·39
D_1	−	−	−	+−	+	+	0·28	0·31
D_2	−	−	−	−	+	+	0·28	0·27
E	−	−	−	+	+−	+	0·18	0·18
F	−	−	−	+−	+	+−	0·09	0·11
G	+−	+−	+−	+	++	++	0·05	0·06
Base line	+−	+−	+	+	+	+	0	0

R_{GA} = 1·0 = distance moved by galacturonic acid. 1—paper irrigated for 70 hours. 2—paper irrigated for 140 hours with the upper layer of *n*-butanol-acetic acid-water (4:1:5 by volume).

Spot size is indicated by the number of (+) signs, (+ −) shows that there was only a trace present, and (−) that no spot was visible. Under the conditions used, arabinose was not separated from fructose, and galactose was not separated from glucose. The single spot D on paper 1 became resolved into two spots on paper 2. Very small spots in the position of xylose (R_{GA} = 1·84) were present in all the juices.

Fructose and glucose are present in rotted tissues and sound tissues in about the same concentrations, whereas sucrose is not present in the rots produced by *S. fructigena*, *B. cinerea*, and *P. furfuracea* although it remains abundant in the *P. expansum* rots. This result is made the more curious by the fact that sucrose added to juices from the first three types of rot did not disappear on incubation.

Little can be said about the unidentified spots at this stage other than to point out that there were more of them in the rots caused by *P. furfuracea* and *P. expansum* than in rots produced by *B. cinerea*, and that, except for spot B which was also present in sound tissue, they were not present in *S. fructigena* rots.

DISCUSSION

The four fungi *Sclerotinia fructigena*, *Botrytis cinerea*, *Pyrenochaeta furfuracea*, and *Penicillium expansum* A were chosen in the first place because they caused rots in apples which were macroscopically quite different; a fifth fungus, *Penicillium expansum* B, was added to see how two isolates of the same species behaved on one type of host tissue. A variety of methods has been used to assess the capacity of micro-organisms to rot structures such as apples.

A popular method in the past has been to remove the rotted tissue and to measure the fresh or dry weight. This is unsatisfactory for a number of reasons, and it may give quite misleading results if it is used to compare different types of rot. A better method is to take the volume of the rot. In many diseases, this can be done readily and the results which are obtained are more easily interpreted than are those based on weighing because the method measures directly the part of the host tissue which has been rotted by the pathogen and this is the important information from the practical point of view. From other points of view, however, it is more important to measure the rate at which the pathogen invades host tissue because this is a better index of its pathogenicity. This rate is not always simply related to the volume of rot produced which depends, *inter alia*, upon the shape of the structure being rotted, and the original position of the inoculum. If the shape of the structure and of the rot are regular and can be defined simply in mathematical terms, and if the position of the inoculum is known, it is possible to estimate the linear rate of advance of the rot from measurements of the rate of increase of the volume of the rot. The linear rate can also be derived from the external dimensions if the rot has a regular and known shape.

When the rate at which each of the fungi moved through apples was estimated it was found that the rates over the first 5 days and 7 days were very similar so that the fungal hyphae grew through the fruit in much the same way as they grew over the surface of an agar which supports good growth. *S. fructigena* advanced about 1·8 times as fast as *B. cinerea*, about 2·2 times as fast as *P. expansum* B, and about 3·0 times as fast as the other two fungi. This rate of radial advance was not related to the type of rot because *S. fructigena*, the fastest, and *P. furfuracea*, the slowest rotting fungus, both produced firm, dark-coloured rots, and *P. expansum* A and *P. furfuracea* which rotted at about the same rate, produced rots quite different in colour and texture. Moreover, the rate at which the fungi grew through apples bore little relation to the rates at which they grew across the surface of apple-extract agar.

On the basis of the rate at which hyphae advanced radially during the first 7 days after inoculation, apples 6·7 cm. diameter would have been rotted by *S. fructigena*, *B. cinerea*, *P. expansum* B, *P. expansum* A, and *P. furfuracea* in 9·6, 16·4, 21·4, 25·4, and 28·0 days respectively. The actual figures in one experiment were 10, 19, 28, 37, and 40 days, but the last two figures are probably inaccurate because for these two rots it was difficult to decide when the apples were fully rotted because small pockets remained unrotted for some time. The extent of the metabolic activity in rotting apples may be gauged from the fact that whereas after 7 days the dry-matter content of all rotted tissues was about the same and only about 5–10 per cent. below that of sound tissue, by the time the fruit had been rotted completely, the dry-matter content for *P. furfuracea* rots had been reduced by about 45 per cent.

In dealing with the pectic substances of sound and rotted tissue, it must be remembered that the best of the methods which have been used so far for extracting these materials from plant tissues do no more than separate them

arbitrarily into a small number of groups on the basis of their solubility in different concentrations of ethanol or acetone, water, dilute acids, and alkalis, and in solutions of substances which sequester polyvalent cations. Solubility in the different solvents will depend primarily upon chain length, degree of esterification, and the extent of cross-linking between adjacent chains. In the native state, other factors may also be important, e.g. linkages between pectic substances and other insoluble components of the cell wall, and physical admixture with other materials which protect the pectic substances from the action of solvents. At present we have little precise information on how the different solvents which are used bring the insoluble pectic substances present in plant tissue into solution, so that the information provided by analyses with a few solvents is necessarily somewhat limited. But in spite of these difficulties, comparison of the data obtained for the different rots shows clearly that each fungus changed the pectic substances present in sound tissue, and did so differently. Even two isolates of the same species did not act similarly in this respect. The most important differences were between the firm, dark-coloured rots in which there was only a 10–20 per cent. loss of total pectic substances, and the soft, light-coloured rots in which up to 70 per cent. was lost. It is not possible to obtain precise figures of loss because these figures depend, to different degrees with different fungi, upon losses of water-soluble pectin, and this hinges upon what is decided to be a pectic substance in terms of solubility in different concentrations of ethanol. *P. expansum* rots contained relatively large quantities of substances soluble in 60 per cent. but insoluble in 95 per cent. ethanol whereas the *S. fructigena* rots did not. A clearer picture of the effects of the fungi would be obtained if a larger number of different concentrations of ethanol were used in the preliminary extractions.

Each of the fungi reduced the quantity of material insoluble in water but soluble in dilute acid, the fraction representing about 95 per cent. of the water-insoluble pectic materials and, therefore, undoubtedly containing a variety of different substances. The firm-rot fungi, *S. fructigena* and *P. furfuracea*, caused relatively little change in this fraction, but the soft-rot fungi reduced it by 50–70 per cent. This fraction insoluble in acid but soluble in dilute alkali is supposed to consist mainly of pectic acid and insoluble pectates and, therefore, to include the middle lamella. In soft rots this fraction is about the same as, or somewhat less than, in sound tissue but in the firm rots it is substantially higher. It must be supposed that the firm-rot fungi alter the solubility of some of the acid-soluble fraction of sound apples, probably by demethylation of pectinic acids to give pectic acid. This aspect will be considered more fully in the second paper, which deals with the pectic enzymes present in sound and rotted tissue.

The most important result from the analysis of water extracts of sound and rotted tissue by paper chromatography was the apparent absence of low molecular-weight polymers of galacturonic acid in *S. fructigena*, *B. cinerea*, and *P. furfuracea* rots, and their presence in *P. expansum* rots in spite of the fact that *P. expansum* degraded the insoluble pectic materials most extensively.

This suggests that the pectic materials of plant tissues are degraded differently by different fungi and this aspect too will be dealt with in the second paper, which will describe the ways in which extracellular enzymes of the fungi act upon well-defined pectic substances *in vitro*.

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Pectic Enzymes and Phenolic Substances in Apples Rotted by Fungi

BY

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Abstract

The activities of pectic enzymes in extracts from sound apples and from apples rotted by different fungi are described. *Sclerotinia fructigena* and *Botrytis cinerea* rots had little or no polygalacturonase or macerating enzyme activity, but *Penicillium expansum* rots were very active in these respects. Extracts from each of the rots had very high pectinesterase activity, and contained galacturonic acid. None of the rots had any cellulase activity. Each of the fungi produced polygalacturonase, macerating enzymes, and pectinesterase in liquid media. The effects of adding extracts of apples to these media are described. Filtrates from cultures of *S. fructigena* and *P. expansum* liberated galacturonic acid from apple fruit fibre which had been thoroughly extracted with cold water.

The phenolic substances present in healthy and rotted tissues were estimated. *B. cinerea* and *S. fructigena* rots contained very little, but *P. expansum* rots contained as much as healthy tissue which had been allowed to brown. An extract of healthy apple tissue reduced the activity of the polygalacturonase in a culture filtrate of *S. fructigena*. The substances responsible for this were tentatively identified as leuco-anthocyanins which had been changed to other compounds following the action of polyphenoloxidase. The significance of these results is discussed.

Introduction

An earlier paper (Cole and Wood, 1961) has described the firm rots in apples caused by *Sclerotinia fructigena* and *Pyrenochaeta furfuracea*, and the soft rots caused by *Botrytis cinerea* and *Penicillium expansum*. Each of these fungi produced changes in the pectic substances of sound apples, but whereas the firm-rot fungi reduced the concentration of water-insoluble pectic substances by up to 15 per cent., the soft-rot fungi reduced it by 50–70 per cent. The pectic substances insoluble in dilute acid but soluble in dilute alkali also were affected differently. The soft-rot fungi either did not affect this fraction or reduced it, whereas the firm-rot fungi caused substantial increases compared with sound tissue.

These results would be explained if the fungi of the two groups produced different quantities and types of pectic enzymes in apples. They would also be explained if there were present in the rots different quantities of substances

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which would reduce enzyme activity or inactivate the enzymes; such substances might be produced by the fungi or by the host as a reaction to infection.

This paper deals mainly with the following topics: (*a*) The types and activities of pectic enzymes which can be extracted from sound apples and from tissues rotted by *Sclerotinia fructigena*, *Botrytis cinerea*, and *Penicillium expansum* (Strains A and B). (*b*) The secretion of pectic enzymes in culture media and how this is affected by adding different types of apple extract. (*c*) The polyphenols present in sound and rotted tissue, and the effect on pectic enzymes of substances produced after oxidation of the polyphenols.

Methods

Some of these are described in an earlier paper (Cole and Wood, 1961). Another pectic substance, sodium polypectate (California Fruit Growers Exchange), was also used after it had been washed in the way described for pectin. The air-dried powder finally obtained was almost white in colour and had a moisture content of 15 per cent.; 1·0 per cent. w./v. solutions had a pH of 5·0. 'Chisel Jersey', a variety of apple with a high tannin content, was kindly supplied by Dr. R. J. W. Byrde and was used as well as Bramley's Seedling in experiments on polyphenols. Methods used to obtain rotted tissue and those used for paper chromatography of sugars and galacturonic acids have already been described (Cole and Wood, 1961). The methods used in the work on polyphenols are described with the experiments.

Extraction of enzymes from apple tissue. The tissues used were incubated for 9 days at 25° after inoculation with *S. fructigena*, and for 16 days at 25° with *B. cinerea* and *P. expansum* A and B; sound tissue was obtained from apples not inoculated but incubated for 16 days at 25°. The tissue was wrapped in several layers of muslin, and the juice extracted in a hand-operated screw press. It was cleared by centrifuging, the pH was adjusted to 5·0 with N-NaOH, and both the juice and the residues from the press were kept at −18°. Later, the frozen residue was thawed and divided into two equal parts. To one was added 0·5 M-Na_2HPO_4, to the other 0·5 M-NaCl at the rate of 1·0 ml. per 1·0 g. fresh weight. The mixtures were stirred at laboratory temperature for 1 hour and the pH of the NaCl solution was kept just above 7·0 by continually adding N-NaOH; the pH of the Na_2HPO_4 solution remained above 7·0. The juice was then extracted in the press, cleared by centrifuging, and the pH was adjusted to 5·0 before the extract was stored at −18°.

All results of experiments in which these extracts were used have been corrected to allow for alterations in volume following addition of NaOH or HCl to adjust the pH; the results for the salt-extracts have been adjusted so that they refer to volumes comparable with the volumes of juice originally obtained from sound or rotted tissue.

Growth of fungi in liquid media. The basic medium was that described by Cole (J. S.) (1956). It contained 1·0 per cent. apple pectin, 1·0 per cent.

ammonium tartrate, 0·1 per cent. KH_2PO_4, 0·05 per cent. $MgSO_4.7H_2O$, adjusted to pH 5·0 before autoclaving at 15 lb./sq. in. for 20 min.

Different types of extract from sound apples were added to the basic medium. 'Unoxidized' apple juice was prepared by pouring boiling water over whole small apples, bringing the water to the boil as rapidly as possible, and boiling for 10 min. After cooling rapidly the juice was extracted from the mixture and cleared by centrifuging to give a pale yellow, syrupy solution which was adjusted to pH 5·0. 'Oxidized' juice was obtained by slicing apple tissue, freezing the slices at −18°, allowing them to thaw, and macerating the pulp in a blender. After 2 hours at laboratory temperature the pulp had become uniformly brown. The juice was then extracted, the pH adjusted to 5·0 and air blown through it for 1 hour. After centrifuging a somewhat turbid, dark brown liquid was obtained. In some experiments, before the 'oxidized' juice was separated from solids the mixture was boiled for 10 min. as was done in the preparation of 'unoxidized' juice. Both types of extracts were sterilized by autoclaving or by passing them through a Chamberlain-Pasteur candle before they were added to the basic medium.

Fungi were grown on the medium containing 2 per cent. w./v. agar and 1 per cent. w./v. glucose in place of the pectin. After 10–12 days at 25° discs, 7 mm. diameter, were cut from the edge of the colony and 5 were placed in 25 ml. of the liquid medium in 8-oz. medicine bottles. The liquid cultures were incubated at 23° for 10 days; they were then strained through muslin and clarified by centrifuging before storage at −18°.

Assay of pectinesterase. 4·0 ml. of the test solution at pH 5·0 (water in the control) were added to 10 ml. 1 per cent. w./v. apple pectin (15 per cent. moisture content) at pH 5·0 and 2·0 ml. N-NaCl, all at 20°. The pH of the mixture was kept near 5·0 by continually adding 0·1 N-NaOH, and activity was measured as the amount of NaOH used in 1 hour after adding the enzyme. Results are expressed as a percentage of the total NaOH required for alkali saponification.

Assay of macerating enzyme. Discs, 0·2 mm. thick, were cut from turgid plugs, 1 cm. diameter, taken from the medulla of a large potato tuber. The discs were washed, drained on blotting-paper, and five were placed in 2·0 ml. of the test solution at pH 5·0 (water in the control) to which had been added 0·5 ml. 0·1-M. citrate buffer at pH 5·0, all at 20°. The time, in minutes, taken for the discs to lose coherence when gently pulled between blunt forceps was obtained and activity is given as the reciprocal of this time × 100.

Assay of polygalacturonase. (*a*) *Reducing group method.* 4·0 ml. of the test solution at pH 5·0 (water in the control) were added to a mixture of 10 ml. 1 per cent. w./v. sodium polypectate (15 per cent. moisture content) at pH 5·0, 4·0 ml. 0·1-M. citrate buffer at pH 5·0, and 4·0 ml. water, all at 20°. Samples were removed immediately and after suitable intervals, and the reducing values were determined by the method of Jansen and MacDonnell (1945) except that, to facilitate mixing, the iodine was added before the carbonate because the latter caused the pectate to gel. Activity is given as the

percentage of total glycosidic groups hydrolysed in the 5 hours following addition of the enzyme.

Assay of polygalacturonase. (*b*) *Viscosity-reduction method.* 1·0 ml. of the test solution at pH 5·0 (water in the control) was added to a mixture of 5·0 ml. 1 per cent. w./v. sodium polypectate (15 per cent. moisture content) at pH 5·0, 2·0 ml. 0·1-M. citrate buffer at pH 5·0 and 2·0 ml. water, all at 25°. The relative viscosity was measured at intervals afterwards in an Ostwald viscometer. Activity is expressed as 100/time taken for the enzyme to produce 50 per cent. of the total possible loss of viscosity. Tests showed that activity measured in this way was directly proportional to enzyme concentration. In some experiments a 1 per cent. w/v apple pectin solution (15 per cent. moisture content) was used in place of the pectate solution.

Results

Activities of Pectic Enzymes in Extracts from Sound and Rotted Tissues

These are summarized in Table 1, which also includes some data from an earlier paper (Cole and Wood, 1961). The activity of the NaCl and Na_2HPO_4 extracts was always of the same order but the phosphate extracts were usually more active. In Table 1 the activity given is the sum of that in the juice and in the more active of the extracts; in brackets is shown the activity of the juice as a percentage of the total. From these figures it will be seen that practically all the pectinesterase in *S. fructigena* rots is adsorbed on the fibre, whereas in the soft-rots produced by *P. expansum* most of the pectinesterase appears in the juice. In the *B. cinerea* rots about two-thirds of the activity is adsorbed on the fibre.

There was little macerating and polygalacturonase activity in the *S. fructigena* and *B. cinerea* rots, so not much importance can be attached to the figures of the proportion adsorbed on the fibre. Juice and extracts of *P. expansum* rots were very active and in five cases between 60 and 78 per cent. of the activity was in the juice; in the sixth the figure was 36 per cent.

No other methods of desorption were used, so it is not known what proportion of the total activities are represented by the figures given in Table 1, but it is probable from the results of other work that little is left on the fibre after extraction with Na_2HPO_4.

Although Glasziou (1958) has produced some evidence that auxin controls the binding of pectinesterase to the cell wall, it is not known what components of the cell wall adsorb this or other pectic enzymes. So at present it is not possible to assess the importance in rotting of the proportion of the enzyme which is bound to the fibre at the low pH of the rotted tissue. However, it may be significant that in the rots caused by *P. expansum*, in which insoluble pectic materials are considerably degraded, most of the activity appears in the juice, whereas in the firm rot produced by *S. fructigena* the only enzyme produced in large quantities is almost completely adsorbed by the fibre, as it is in sound tissue. In the rest of this paper only the total enzyme activity in rotted tissue will be considered.

TABLE 1

Activities of Pectic Enzymes in Extracts from Tissue of Sound and Rotted Apples

	Pectinesterase	Macerating activity	Polygalacturonase (reducing) polypectate	Polygalacturonase (viscosity)		Reaction products†								Reaction products in rotted apple tissue‡		Rate of linear advance‡	% loss of insoluble pectic substances‡
						Polypectate substrate				Pectin substrate							
						Juice		Phosphate extract of fibre		Juice		Phosphate extract of fibre					
				Polypectate	Pectin	GA	nGA	GA	nGA	GA	nGA	GA	nGA	GA	nGA		
Sound apple	5 (23)*	o (—)	o (—)	o (—)	o (—)	o	o	o	o	o	o	o	o	o	o	o	o
S. fructigena rot	67 (2)	o (—)	0·2 (100)	0·5 (0)	v.l. (0)	o	o	o	o	o	o	o	o	+	o	0·29	13
B. cinerea rot	47 (35)	v.l. (—)	3·7 (0)	4·0 (25)	v.l. (0)	++	o	++	tr.	++	o	+++	o	+++	o	0·17	51
P. expansum A rot	29 (91)	6·2 (74)	24·2 (69)	151·0 (36)	5·0 (60)	+++	++	+	+++	+	+	++	tr.	++	+	0·11	52
P. expansum B rot	24 (78)	12·5 (73)	34·2 (64)	3600·0 (66)	21·2 (78)	+++	+++	++	+++	++	++	++	tr.	+++	+	0·13	71

* Total activity for whole rot (i.e. juice and fibre). The figures in brackets represent the activity of the juice as a percentage of the total activity.
† Juice or extract, plus substrate, incubated at 25° for 24 hours. Composition of reaction mixtures as in assay of polygalacturonase (viscosity).
‡ Data taken from Cole and Wood (1961).
GA = galacturonic acid. nGA = di- and tri-GA.
tr. = a trace.
v.l. = very low.

S. fructigena rot. Although total pectinesterase activity was very high most of it was adsorbed on the fibre. About two-thirds of the methoxyl groups in the test solution were de-esterified in the first hour at a rate directly proportional to time. Afterwards the rate of de-esterification fell off rapidly and the reaction was virtually complete after 3 hours. In the succeeding 16 hours the average rate was only about 1/200 that in the first hour, so that after 19 hours de-esterification by the enzyme could be regarded as complete. When more substrate was added acid groups were liberated rapidly so that the enzyme was still active. Nevertheless, de-esterification of the first batch of substrate had stopped when it still contained about 10 per cent. of the total methoxyl groups which could be liberated by alkali. Similar results have been obtained by other workers (Kertesz, 1951, p. 129).

There was no macerating activity in juice or extracts and little polygalacturonase activity even when this was measured by the sensitive viscosity method. The very low activity was surprising in view of the fact that galacturonic acid was readily detected in the juice from rotted tissue, and that about 13 per cent. of the insoluble pectic material of sound tissue was degraded. Also, it is generally found that when pectinesterase is produced freely by a fungus, chain-splitting enzymes are also produced. The results obtained for this type of rot would be more readily understood if it could be shown that the fungus did produce chain-splitting enzymes in rotted tissue and that these enzymes were inactivated afterwards. This possibility will be discussed later.

Rotted tissue, compared with sound tissue, contains more pectic substances soluble in dilute alkali but insoluble in dilute acid, that is, more of substances with a low methoxyl content. A possible explanation of this is that the fungus secretes enzymes which produce pectinic acids from a small part of the protopectin. These pectinic acids are not extensively degraded because the polygalacturonases are inactivated, but they are de-esterified by the active pectinesterase produced by the fungus, with the result that pectates insoluble in dilute acid but soluble in dilute alkali are produced.

B. cinerea rot. Again there was a high pectinesterase activity in the rot and most of it was retained by the fibre. There was little macerating activity and rather low polygalacturonase activity when polypectate was the substrate. The activity was still lower when pectin was used. These results were unexpected for several reasons. Firstly, the fungus degraded about half the insoluble pectic substances originally present in sound tissue. Secondly, galacturonic acid was present in relatively large amounts in rotted tissue and in solutions of polypectate and pectin which had been incubated with the enzyme. Thirdly, rotted tissue contained relatively large quantities of pectinesterase, so it is surprising that pectin was not degraded more rapidly.

Tentatively, it is suggested that the fungus produces in rotted tissue an exopolygalacturonase (Demain and Phaff, 1957) which acts upon soluble pectates by removing end units of galacturonic acid.

P. expansum rots. Substantial quantities of pectinesterase were produced

but this time most of the enzyme was in the juice. In contrast with the other rots, there was abundant macerating and polygalacturonase activity in each of the rots, and most of the activity was in the juice. Strain B, which degrades more of the insoluble pectic substances than does strain A, also produces more of the chain-splitting enzymes. Both strains produce large quantities of galacturonic acid and short chain polymers of this acid in rotted tissue and in digests of polypectate and pectin; and the polygalacturonases produced are much more active on polypectate than on pectin. By the reducing group assay method, strain B is somewhat more active than strain A, but by the viscosity method it is very much more active both on polypectate and on pectin. These results were confirmed a number of times but no explanation for them can be given at present.

Cellulase Activity

Neither juice, nor salt extracts of sound and rotted tissue reduced the viscosity of solutions of sodium carboxymethylcellulose (degree of substitution 0·7) at 25° and pH 5·0 and no products of hydrolysis were detectable by paper chromatography.

Activity of Enzymes Produced in Liquid Culture

The fungi were grown in the synthetic medium ±1, 10 or 50 per cent. v./v. apple juice, and in 50 per cent. v./v. apple juice; both oxidized and unoxidized apple juice were used. The pectinesterase, macerating, and polygalacturonase (viscosity method on polypectate) activities, and the pH and colour of the culture filtrates were measured after 10 days' growth at 23°.

The main object of the experiment was to compare the effect of the unoxidized and oxidized polyphenols of apple juice on the secretion of pectic enzymes by the fungi, and on the activity of these enzymes after secretion. This object was not achieved because media to which unoxidized juice was added became brown on autoclaving, and this difficulty was not avoided when media were sterilized by filtration; in fact such media became even browner on standing. Another difficulty was that *S. fructigena* and *B. cinerea* secrete polyphenoloxidase during growth so that, presumably, some of the polyphenols remaining in unoxidized juice would be oxidized during incubation.

Because of these complications no clear-cut results were obtained as to the effects of adding oxidized or unoxidized juice to the media, so this aspect will not be considered further. However, a number of points did emerge from the experiments as will be seen from the data summarized in Table 2, which relates to media containing unoxidized juice. Each figure is the mean of the results obtained with duplicate cultures; the individual results always agreed quite closely. Because earlier results had shown that the two strains of *P. expansum* behaved similarly, only strain A was used in these experiments.

Filtrates from cultures of *S. fructigena* in synthetic media, with or without apple juice, had a fairly high polygalacturonase activity and there were indications that this activity was reduced as the proportion of juice increased.

TABLE 2

Activity of Enzymes Produced in Liquid Culture

	Enzyme	Enzyme activity in various media: % v./v. unoxidized juice added to basal medium				50% v./v. unoxidized juice in water
		0%	1%	10%	50%	
S. fructigena	Pectinesterase	23	30	26	60	9
	Macerating	1·2	2·1	1·7	1·0	1·0
	Polygalacturonase*	48	48	39	22	0
B. cinerea	Pectinesterase	4	6	6	8	8
	Macerating	1·7	1·7	1·7	1·5	0·5
	Polygalacturonase*	7	12	20	29	0
P. expansum A	Pectinesterase	0	0	trace	trace	4
	Macerating	3·6	3·7	9·1	14·3	0·6
	Polygalacturonase*	0	0	trace	trace	4·5

* Viscosity method, polypectate substrate.

Filtrates from cultures grown on apple juice alone were inactive. Pectinesterase activity was highest in the medium containing synthetic constituents and the highest concentration of apple juice; it was much lower when juice alone was used. Macerating activity was reduced when the proportion of juice was increased but there was still some activity after growth on the juice alone.

The composition of the media had little effect on pectinesterase and macerating activities of *B. cinerea* filtrates but the addition of the synthetic constituents increased the activity of polygalacturonase enzymes in the filtrates.

Filtrates from cultures of *P. expansum* contained pectinesterase only when the medium contained 50 per cent. juice; synthetic constituents had little effect. All filtrates from cultures on synthetic media had a high macerating activity but very low or no polygalacturonase activity. This result was confirmed a number of times and is the more surprising because the juice and extracts from tissue rotted by this fungus had a high macerating *and* polygalacturonase activity. Further tests showed that the filtrates with high macerating activity did not reduce the viscosity of solutions of pectate derived from pectin by de-esterification with pectinesterase from tobacco leaves, but that the viscosity of pectin solutions was rapidly reduced. These results are in direct contrast with those given in Table 1 where the polygalacturonase produced in rotted tissue was considerably more active on polypectate than on pectin. In culture filtrates, therefore, high macerating activity was associated with high endopolymethylgalacturonase (Demain and Phaff, 1957), whereas in rotted tissue the association was with high endopolygalacturonase activity.

The data in Table 2 also show that the level of macerating activity is not always directly related to the level of polygalacturonase activity taken alone or together with pectinesterase activity.

Paper chromatography showed that whereas mono- and polygalacturonic acids were not detected in any of the culture filtrates, both were detected after polypectate and pectin had been incubated with filtrates from cultures of *S. fructigena* and *B. cinerea*. Only monogalacturonic acid was found when *P. expansum* A filtrates were used.

Action of Culture Filtrates on Apple Fibre

Filtrates from cultures of *S. fructigena* and *P. expansum* A with the highest macerating activity were used. The fibre was prepared by freezing apples quickly, slicing them into water at 0°, and macerating the mixture in a blender. The mixture was filtered and the residue washed repeatedly in water until no sugars could be detected when a drop of the liquid in which the fibre was suspended was placed on filter-paper and examined by paper chromatography. The fibre was then dried in the frozen state to give a pale-brown spongy mass which was readily broken down to a powder; 0·15 g. of this powder was suspended in 4 ml. of the culture filtrates at pH 5·0 or 4 ml. water and at intervals samples were removed for examination by paper chromatography. Substances already present in the filtrates were allowed for in assessing the results.

In the controls in which the fibre was suspended in water only, no sugars were detected after incubation for 130 min.; after the same time a definite galacturonic acid spot was obtained from the mixtures containing *S. fructigena* filtrates. With the *P. expansum* filtrate an equally prominent spot was obtained after 20 min.; in later samples the spot was larger and a number of more slowly moving spots also appeared. After 60 min. there was a prominent spot in the arabinose position and a trace of a spot in the galactose position; both of these spots were more prominent after 130 min.

The *P. expansum* filtrate had a low polygalacturonase activity when polypectate was the substrate, but high when pectin was the substrate, and a low pectinesterase activity, so that the results suggest that it was the high methoxyl pectic substances of the cell wall which were being degraded during maceration.

Extraction and Estimation of Total Phenols in Sound and Rotted Tissue

Sound and rotted apples were frozen at −18°. Two 10 g. samples were taken from just below the surface of rots caused by each fungus. The samples were ground finely at −18° and then 30 ml. boiling ethanol (95 per cent. v./v.) were added to each and the mixture boiled for 1·5 min. The suspension was cooled, allowed to stand for 2–3 hours, centrifuged, and the supernatant transferred to a 100 ml. flask. The residue was extracted a number of times with ethanol, the extracts were combined, made up to 100 ml., and stored at 4° until required. Four samples of sound tissue were

dealt with similarly except that two of them were allowed to stand at room temperature for 30 min. before extraction with ethanol. The preparations from the Chisel Jersey apples browned much more rapidly than did those from Bramley's Seedling.

The total phenols in the ethanol extracts were estimated by the method of Swain and Hillis (1959), which is based on the Folin–Denis reagent. When (+)-catechin was used a linear relationship was obtained between optical density at 725 mμ and concentration over the range 0–50 μg./ml. The total phenolic content of the tissue is expressed in terms of (+)-catechin. The results are shown in Table 3.

TABLE 3

Total Phenols in Sound and Rotted Apples

	Variety of apple	
	Bramley's Seedling	Chisel Jersey
Sound apple	18·7 (100)	41·6 (100)
Sound apple after browning	8·8 (47)	22·3 (54)
S. fructigena rot	4·0 (21)	3·2 (8)
B. cinerea rot	5·6 (30)	4·5 (11)
P. expansum A rot	8·1 (43)	21·1 (51)

Figures shown outside brackets are mg. total phenols as (+)-catechin/g. dry wt. of sound apple, or sound apple invaded by the fungus.

Leuco-anthocyanins and flavanols in the tissue extracts were also estimated by the methods described by Swain and Hillis (1959). The results showed that these groups of substances were affected during rotting in much the same way as the total phenols.

When sound tissue of both varieties of apple was disintegrated and allowed to stand for 30 min. it became brown and about 50 per cent. of the soluble phenols disappeared. The concentration of phenols present after this treatment was about the same as that present in tissue rotted by *P. expansum* A which, in Bramley's Seedling, was almost white in colour. These colour differences for similar phenol losses indicate that in Bramley's Seedling *P. expansum* has a mechanism for converting or destroying the host phenols, without polymerizing them into brown-coloured compounds, which would involve preventing the action of host phenolase. In Chisel Jersey a brown-coloured rot is produced by *P. expansum* which suggests that either the above mechanism is not operating or that the level of host phenols and phenolases is so high that some brown-coloured compounds are formed in spite of it.

In rots of Bramley's Seedling fruit caused by *B. cinerea* and *S. fructigena* the concentration of phenols was much lower than in *P. expansum* rots, and in Chisel Jersey the concentrations were still lower, although the fruit at first contained more than twice as much as did Bramley's Seedling.

When agar media containing pyrocatechol or gallic acid were inoculated with the three fungi, *B. cinerea* and *S. fructigena* caused intense browning of the media, *S. fructigena* causing more browning in the gallic acid medium and

B. cinerea more in the pyrocatechol medium. *P. expansum*, although it grew on both media, failed to cause browning of either substrate, which suggests that it does not produce polyphenoloxidase. This may in part explain why rots caused by this fungus contain more soluble phenols than those caused by the other two, although polyphenoloxidase must have been present at some stage in all the rots because it is present in the cells of the apple tissue.

Chromatography of Phenols in Sound and Rotted Tissue

The ethanol extracts of the Bramley's Seedling fruit prepared as described in the previous section were analysed by two-dimensional paper chromatography to see which types of phenols were affected during rotting. Chlorogenic acid and catechin were used as markers. Four ml. of ethanol extract were concentrated at 35° under reduced pressure and the concentrate transferred to a sheet of Whatman no. 20 paper. This was irrigated in the first dimension with the upper layer of an *n*-butanol–acetic acid–water (4:1:5 by vol.) mixture for 48 hours at 20° and in the second dimension with 2 per cent. v./v. aqueous acetic acid for 18 hours at 20° (Cartwright and Roberts, 1954). The sheet was dried at room temperature and examined first under u.v. light, and then under u.v. light in the presence of ammonia vapour. All fluorescent or absorption spots were marked. Spots corresponding to chlorogenic acid were cut out and treated in a way to be described later. The rest of the paper was sprayed with a freshly prepared mixture of equal volumes of 1 per cent. w./v. ferric chloride and 1 per cent. w./v. potassium ferricyanide (Whiting and Carr, 1957). Immediately afterwards the sheets were washed successively in 0·1 per cent. v./v. HCl, water, 95 per cent. v./v. ethanol, and left to dry at room temperature.

The above tests revealed the presence of a number of phenols (Table 4), some of which have already been reported as being present in sound apple tissue (Cartwright, Roberts, Flood, and Williams, 1955; Hulme, 1953; Williams, 1953).

TABLE 4

Phenols in Sound and Rotted Apples

	Sound tissue	Tissue rotted by		
		S. fructigena	*B. cinerea*	*P. expansum* A
Chlorogenic acid	+++	+	trace	+
p-Coumarylquinic acids	++	+	—	—
Catechin and epicatechin	+++	—	—	—
Leuco-anthocyanins	+++	trace	trace	trace
Caffeic acid	—	+	—	+
7, 17, 18, 19 (unidentified chromatogram spots*)	—	+	+	+

* Spots 17, 18, and 19 were found as a group with R_f 0·5–0·7 in the first dimension (top layer *n*-butanol–acetic acid–water, 4:1:5 by volume) and 0–0·2 in the second dimension (2 per cent. acetic acid). Spot 7 had an R_f of about 0·7 in first dimension and about 0·4 in the second.

The chromatograms showed clearly that each of the fungi caused the disappearance of the catechins, most of the leuco-anthocyanins, and a large part of the chlorogenic acid. They also caused the appearance of a substance tentatively identified as caffeic acid which could have arisen by the hydrolysis of chlorogenic acid, and four compounds which were not identified. *p*-Coumarylquinic acids were present in sound tissue, and in tissue rotted by *S. fructigena*, but not by *B. cinerea* or *P. expansum* A.

The parts of the paper containing chlorogenic acids (*cis* and *trans*) were eluted with water (before spraying) and the amounts of the acids in the eluates were determined by optical density at 325 mμ compared with those given by known concentrations of chlorogenic acid chromatographed in the same way. If the concentration of chlorogenic acid in sound tissue is taken as 100, the concentration in the different extracts was as follows: browned but not rotted tissue, 92; *S. fructigena* rot, 12; *B. cinerea* rot, 1; *P. expansum* A rot, 11. From these figures it is clear that it is the fungi which cause the almost complete loss of chlorogenic acid from rotted tissues, either by acting upon them directly or by increasing the activity of the oxidizing enzymes present in the host cells.

Interactions between Phenolic Substances and Pectic Enzymes

The above evidence suggested that the differences between the rotted tissues as regards pectic enzymes and degradation of pectic substances might be related in some way to the effects of polyphenols, or products arising from their oxidation, on pectic enzymes secreted by the pathogens. Because the most striking results which had been obtained were the absence of chain-splitting enzymes, and the limited degradation of pectic substances, in *S. fructigena* rots, only the enzymes secreted by this fungus were considered initially.

The fungus was grown on the synthetic medium described earlier, and cell-free extracts were obtained which rapidly reduced the viscosity of solutions of sodium polypectate. This enzyme solution was incubated with different phenols, and with different extracts from apple in attempts to identify materials which might cause inactivation of chain-splitting enzymes *in vivo*.

The presence of a polygalacturonase inhibitor in sound apple was demonstrated in the following experiment. Samples of the alcohol extracts of healthy apples, healthy browned apples, and *S. fructigena*-rotted apples were evaporated to small volumes to remove the alcohol and diluted with water to a final concentration of 5× original. The solutions so obtained were incubated for 3 to 5 hours, ±NaCN, with 0·5 volumes *S. fructigena* enzyme solution and then the polygalacturonase activity was measured viscometrically. The only reaction mixture in which a reduction (70 per cent.) of enzyme activity occurred was that containing healthy-apple extract in the absence of NaCN; this mixture turned brown during the incubation. NaCN (final conc. 0·005 M.) prevented the inhibition of polygalacturonase. The extracts of browned healthy apple and *S. fructigena*-rotted apple contained no inhibitor

possibly because it was made insoluble during browning and was therefore not extracted.

The alcohol extract of sound apples contained chlorogenic acid and (+)-catechin but the following experiment suggests that neither of these substances acted as inhibitors. Mixtures containing 40 per cent. (v./v.) enzyme solutions, 40 per cent. (v./v.) of a solution of chlorogenic acid (0·1 mg./ml.) or catechin (0·05 mg./ml.), and 20 per cent. (v./v.) 0·02 M. NaCN or water were incubated for 2 hours at 20° and then 1 ml. was transferred to the polypectate/buffer solutions already described. None of the substances in the presence or absence of NaCN had any effect on the activity of the enzyme which reduced the viscosity of the polypectate solution. Further tests showed that these two substances also had no effect on the macerating enzyme in the culture filtrates.

Isolation of Inhibitor by Paper Chromatography

A 10 ml. sample of the ethanol extract of sound apple was concentrated to a small volume under reduced pressure at 35°, and the whole volume was distributed between eight points 2 cm. apart along a line 9 cm. from the top of a sheet of Whatman no. 20 paper. The paper was irrigated with 2 per cent. (v./v.) acetic acid in water for 12 hours at 20°. The solvent front was marked and the sheet was dried at room temperature. After the limits of the fluorescent zone caused by chlorogenic acid were marked under u.v. light two strips, associated with the outer two of the eight points, were removed, one was sprayed with the ferric chloride-potassium ferricyanide reagent, and the other was sprayed with 2N-NaOH and examined under u.v. light. The first strip showed a continuous band of phenolic material down to the lower chlorogenic acid zone, and the second strip revealed chlorogenic acid as two yellow spots.

The remaining centre strip was divided into six horizontal bands each 4 cm. wide and the phenolic materials in each band were eluted in 1 ml. water; only traces of phenols remained on the paper after this treatment. Each of the volumes obtained was incubated with 1 ml. of an *S. fructigena* enzyme solution for 7 hours at room temperature before measuring activity against a solution of polypectate. Only the eluate from the fourth band from the top (R_f of mid-point = 0·37) affected enzyme activity, causing a reduction of 25 per cent. This band contained the lower of the chlorogenic acid spots but because it had already been found that chlorogenic acid had no effect on enzyme activity it seemed that some other substance of similar R_f was reducing enzyme activity; reference to the two-way chromatograms indicated that this might be leuco-anthocyanin. The effects which had been obtained were small because there would be very little of the inhibitor on the paper, so larger quantities were obtained in the following way.

Extraction and Activity of Phenolic Compounds of Sound Apple

The method used to extract and separate the polyphenols in sound apples was based in part on a procedure described by Roberts, Cartwright, and

Wood (1956) for isolating leuco-anthocyanins from tea leaf and is summarized below.

Two hundred g. fresh-apple tissue were extracted three times to give 400 ml. ethanol extract. This was concentrated to 10 ml., diluted with 25 ml. methanol, and extracted with 175 ml. of chloroform to give a chloroform extract (1) and a precipitate. The precipitate was washed in chloroform and dissolved in 50 ml. of water which was then extracted with 2×50 ml. ethyl acetate to give an acetate extract (2) and an aqueous layer. The aqueous layer was extracted with 3×15 ml. *n*-butanol to give a butanol extract and an aqueous layer. The butanol extract was evaporated at 60° to give a residue which was dissolved in 5 ml. methanol. The addition of 50 ml. of ethyl acetate to this solution gave a precipitate which was dissolved in 5 ml. methanol (3). The ethyl acetate solution was concentrated and a precipitate which formed early during the process was added to (3) before the solution was finally evaporated to dryness. The residue obtained was washed with ethyl acetate and dissolved in a mixture of 10 ml. ethyl acetate and 2·5 ml. methanol (4).

Samples corresponding to one-fifth to one-tenth of extracts 1, 2, 3, and 4 were evaporated to dryness under reduced pressure and the residues dissolved in 1·5 ml. water; 0·02 ml. of the aqueous solutions were applied to Whatman no. 1 paper which was irrigated with the upper layer of an *n*-butanol–acetic acid–water (4:1:5 by vol.) mixture for 18 hours at 18°, dried, and treated in various ways to identify the phenolic substances present. Samples (0·5 ml.) of the aqueous extracts were incubated with 0·5 ml. of the *S. fructigena* enzyme solution $\pm$ NaCN at a final concentration of 0·01 M., for 10 hours in the dark at 20°. Polypectate solution and buffer were then added to each of the mixtures and enzyme activity was measured by the viscosity method. After these measurements had been made, the solutions were incubated under toluene for 14 hours at 20° and samples were analysed by paper chromatography for galacturonic acid and its low molecular-weight polymers. The results shown in Table 5 allow for the fact that different volumes of the total extracts were used for preparing the aqueous solutions.

Fraction 1 containing chlorogenic and *p*-coumarylquinic acids was virtually inactive. The data from Table 5, together with the results from the chromatograms, showed that for fractions 2, 3, and 4 in the absence of NaCN reduction of activity corresponded with the presence of readily detectable polyphenols with R_f between 0·2 and 0·4. When NaCN was present, fractions 3 and 4 still reduced activity and in these fractions there were relatively large quantities of polyphenols with R_f 0 to 0·2.

Reaction mixtures containing fractions 1, 2, and 3 darkened in colour compared with the same solutions containing NaCN probably because of the action of polyphenoloxidase. The presence of polyphenoloxidase in the *S. fructigena* enzyme solution was also inferred because solutions of pyrocatechol, (+)-catechin, gallic acid, tannic acid, and chlorogenic acid turned brown or buff-coloured in the presence of the enzyme solution, and because

TABLE 5

Effect of Phenolic Fractions on Enzyme Activity

Phenolic fraction	Phenols present	NaCN absent (—) present (+)	% reduction in activity compared with controls*
1	A(++) B(++)	—	13
		+	2
2	A(++) B(+) C(+++) D(++)	—	55
		+	9
3	D(++) E(+++)	—	63
		+	44
4	A(++) B(++) C(+) D(+++) E(+)	—	88
		+	42

* Reduction in activity given by a volume of aqueous extract equal to 5 per cent. of total extract.

A = chlorogenic acid
B = *p*-coumarylquinic acid
C = catechins
D = leuco-anthocyanins of R_f 0·2—0·4
E = leuco-anthocyanins of R_f 0—0·2

the enzyme solution caused the disappearance of soluble phenols from apple extract when examined chromatographically, but this was prevented in the presence of NaCN.

Chromatograms showed that fraction 4, in the absence of cyanide, caused a marked reduction in the amount of mono-, di-, and tri-galacturonic acids which were produced from the reaction between enzyme and polypectate. Reductions were also obtained in the other mixtures where the activity of the enzyme had been lowered, but none was so striking.

Samples of aqueous solutions of fractions 2, 3, and 4 were analysed chromatographically in an attempt to identify the components which were reducing enzyme activity. After papers had been irrigated (upper layer of *n*-butanol–acetic acid–water, 4:1:5 by vol., 22 hours, 18°, Whatman no. 1) and air dried they were: (*a*) examined in u.v. light with and without ammonia vapour, then sprayed with 2N-NaOH and examined in daylight and u.v. light (Swain, 1953); (*b*) sprayed with diazotized *p*-nitroaniline in sodium acetate solution; (*c*) sprayed with a vanillin-HCl reagent (Bate-Smith, 1954; Swain and Hillis, 1959); (*d*) extracted with acid butanol at 100° and the pink coloration measured (Swain and Hillis, 1959).

The results showed that the substances with R_f 0 to 0·2 and 0·2 to 0·4 were leuco-anthocyanin in nature (Table 5). Where these substances were present enzyme activity against polypectate was reduced in the absence of NaCN. When NaCN was present reduction of activity was most marked when the fraction contained the very low R_f leuco-anthocyanins. As indicated in a preliminary report (Cole, 1958) these results suggest that the low R_f leuco-

anthocyanins were inhibitors, active in the presence of NaCN, and that the higher R_f leuco-anthocyanins only became inhibitory after oxidation (probably to the lower R_f form) by fungal polyphenoloxidase, a process which takes place only in the absence of cyanide (see fraction 2, Table 5).

Discussion

Each of the three fungi used in this investigation, *S. fructigena*, *P. expansum*, and *B. cinerea*, produced in liquid culture macerating enzymes, pectinesterase, and polygalacturonase. However, when juice was extracted from tissues rotted by these three fungi or the residues were extracted with salt solutions that would be expected to release enzymes adsorbed by host tissue, it was found that juices and extracts from rots caused by *S. fructigena* and *B. cinerea* had very little macerating or polygalacturonase activity. For *S. fructigena* these results were not unexpected because it had been found that there was not much loss of total pectic substances in rotted tissue, but the low activity of the *B. cinerea* extracts was surprising because of the soft consistency of the rot and because in rots caused by this fungus about 40 per cent. of the total, and 50 per cent. of the insoluble, pectic substances of the healthy tissue were degraded. Galacturonic acid was readily detected in the juice from tissue rotted by all three fungi and this was strong evidence that pectic enzymes had been active, as this acid is absent from healthy apple tissue.

The high macerating and polygalacturonase activity of *P. expansum* rots corresponded with the considerable loss of soluble and insoluble pectic substances originally present in healthy tissue, with the presence of galacturonic acid in rotted tissue, and with the softness of the rot. Extracts of each of the three rots had high pectinesterase activity. In the firm rots caused by *S. fructigena* most of the enzyme is adsorbed by host tissue and is not extracted with water. Although the *B. cinerea* rot is quite soft, about two-thirds of the enzyme is adsorbed, whereas in the soft rot caused by *P. expansum* most of the enzyme is found in the juice. *S. fructigena* rots were also characterized by an increase, relative to healthy tissue, of pectic substances insoluble in dilute acid, i.e. pectic acid or pectates. This can be explained by assuming that enzymes produced by the fungus act upon pectic substances of the host for a short time only and that the pectinic acids which are released are de-esterified by the pectinesterase produced by the fungus to give insoluble compounds.

P. expansum rots and culture filtrates had high macerating and polygalacturonase activity, but the polygalacturonases of the two preparations acted differently. The enzyme preparation from rots contained pectinesterase and degraded polypectate much more readily than pectin. This has been the usual result with other fungi and is typical of the enzyme endopolygalacturonase. The enzyme produced *in vitro* was inactive on polypectate and on pectate derived from the pectin, but, although it contained no pectinesterase, it degraded pectin. In this respect the enzyme behaved like endopoly-

methylgalacturonase (Demain and Phaff, 1957). If different enzymes are produced under the two conditions, then both types of polygalacturonase were associated with an ability to macerate plant tissue. At present the significance of this cannot be assessed because of our ignorance of what components of the cell wall are degraded during maceration.

Differences in pectic enzyme content were associated with differences in the colours of the rots which would depend upon the activity of phenolases on phenols present in host tissue. Analysis showed that in the coloured rots caused by *B. cinerea* and *S. fructigena*, the concentration of soluble phenols was much less than in the white rot caused by *P. expansum*. Coloration and low content of soluble phenols was, therefore, associated with low concentration of chain-splitting pectic enzymes. For *S. fructigena* it was shown that products formed on the oxidation of some of the phenols present in host tissue, probably the leuco-anthocyanins, inactivated the polygalacturonase of this fungus; NaCN prevented this inactivation.

Tentatively the following scheme can be proposed for the *S. fructigena* rot. The fungus begins to grow into healthy apple tissue and starts to produce pectinesterase and chain-splitting enzymes. The latter cause some degradation of cell-wall pectic substances and the pectinesterase de-esterifies some of the high molecular-weight pectinates to form pectates. The cells of the host are killed, possibly as a result of the action of pectic enzymes on the cell walls, and phenols are brought together with host and fungus phenolases to form coloured oxidation products some of which inactivate the chain-splitting enzymes. If *these* enzymes are produced only by young, actively growing hyphae at the advancing edge of the lesion, the degradation of pectic substances would be limited to this zone and enzymes left behind by the growing hyphae would be inactivated so that the bulk of the pectic substances in the rotted area would not be attacked.

In *B. cinerea* rots there may be similar inhibitors, but if they are present they must be produced less rapidly or act more slowly, because although there are no endopolygalacturonases in the rots, the extensive degradation of pectic substances shows that they must have been produced and active for some time afterwards. Some results suggested that an active exopolygalacturonase enzyme (Demain and Phaff, 1957) was present in *B. cinerea* rots because although extracts from rots showed very low activity against pectin and pectate by the viscosity method, galacturonic acid was readily formed from these substrates. This exopolygalacturonase enzyme must have been unaffected by the factors which destroyed the activity of the other polygalacturonase enzymes.

The picture for *P. expansum* rots is quite different. If inhibitors are produced here there is no evidence to show that they affect the enzymes of this fungus. It is more likely that there is present in these rots a system which prevents the formation of the type of inhibitor found in *S. fructigena* rots because the rots in Bramley's Seedling are white in colour and have, relative to the rots caused by the other fungi, a higher soluble phenol content.

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We should like to thank Dr. A. C. Hulme for his valuable advice and one of us (M. C.) wishes to thank the Department of Scientific and Industrial Research for a Research Studentship.

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The Effect of Gibberellic Acid on Fibre-cell Length

BY

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With five Figures in the Text

Abstract

Measurements were made of fibre-cells from plants of *Corchorus olitorius* L., *Hibiscus cannabinus* L., and *Cannabis sativa* L. which had been sprayed with gibberellic-acid solution. Fibre-cells from treated plants showed a highly significant increase in length, 20–130 per cent. for the whole stem and as much as 400 per cent. for a single internode.

Gibberellic acid increased the variation in cell-length and the positive skewness of the distribution of the variate.

Differences in cell-length can be related to the developmental sequence of the shoot and the variation in internode-length.

Introduction

GIBBERELLIC acid produces striking effects on plant growth (Brian, 1959), the most characteristic of which is a great increase in plant height which can be apportioned into longitudinal extension of its separate morphological components. The question arises whether these spectacular increases in length are due to increase in cell-number or cell-size. There has been a considerable amount of work on this problem producing evidence in support of both hypotheses (Greulach and Haesloop, 1958; Kato, 1955). It is now generally accepted that gibberellic acid probably affects cell-expansion and cell-division (Guttridge and Thompson, 1959; Humphries and Wheeler, 1960). Sachs and Lang (1957) suggest that gibberellic acid may function as a regulator of cell-divisions and elongation, mitoses possibly being induced indirectly by the acceleration of cell-extension.

In the present work an attempt is made to determine whether gibberellic acid affects the longitudinal extension of the long fibre-cells of some fibre-producing plants.

A range of plants was grown at Kew to study their reactions to gibberellic acid. A number of fibre-producing plants of economic interest was included in the study, and the following species will be referred to in this paper, *Corchorus olitorius* L., *Hibiscus cannabinus* L., and *Cannabis sativa* L.

Materials and Methods

Plants for the experiment were grown specially from seed and divided into two sets when a few inches high. A control set of ten plants of each species was sprayed weekly with distilled water. The duplicate set of plants was given a weekly treatment with 100 p.p.m. solution of gibberellic acid in

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water containing a wetting agent. The solution was applied as a fine spray with an atomizer until the leaf surfaces became thoroughly wet.

Quantitative observations of differences in growth and development between the two sets of plants were made throughout the period of the experiment and will be described in a separate paper. Gibberellic acid induced great increases in plant height and internode length, and from this material measurements of fibre-cell length were made to ascertain whether the treatment had affected the dimensions of these cells from the stem.

At the end of the experiment, the stems of treated and control plants were cut through transversely at each node. The separate internodes were then left to ret in tap water for several months. It was necessary to change the water occasionally. When a certain stage of softness is reached (the length of this period varies in the different species), it is possible to strip away the outer layers from the wood, which can then be removed as a solid cylinder, leaving a hollow cylinder comprising the peripheral tissues. This outer cylinder was returned to the retting tubes and the process allowed to continue until the fibres could be disentangled from the softer tissue in which they are embedded. At this stage the epidermis and outer cortex are easily stripped off.

To obtain individual fibre-cells, the long bundles of fibres were macerated for 12–24 hours in a mixture of 5 per cent. nitric acid and 5 per cent. chromic acid (equal parts by volume). It was convenient to leave the fibres in the macerating fluid overnight and remove samples the next morning to test for degree of separation of the cells. If the process was not complete, a second sample could be examined after another hour, and so on. It is necessary to arrest the maceration at exactly the right stage of disintegration in order to obtain single fibre-cells which are fairly straight and undamaged. This can only be ascertained by taking test samples at hourly intervals. Wherever the procedure was very protracted, the separation was accelerated by placing the maceration tubes in an oven at 55° C.

After washing several times in tap water, the fibre-cells can be teased apart on the stage of a binocular dissecting microscope. Temporary mounts in glycerine were made. These slides were projected and linear measurements of the cells made with a ruler at an appropriate magnification. ×35 was found to be suitable for most of the cells. One hundred readings were taken for each internode. This is a convenient number to deal with and was considered a large enough sample to provide statistical proof of any differences that were noted. Significance was based on Student's *t* test.

Results

All plants which had received treatment with gibberellic acid showed a significant increase in fibre-cell length compared with similar cells from control plants. Results are summarized in Table 1 which gives the mean length of fibre-cells from all parts of the stem.

In Table 2 a comparison is made between mean lengths of fibre-cells from

the sixth internodes (internodes were numbered from the base of the stem upwards) of control and treated plants. Gibberellic acid induces increases in fibre-cell length of the order of 20–150 per cent. The frequency distribution of the readings of cell-length averaged in Table 2 is given in Fig. 1 for *Hibiscus cannabinus* and *Corchorus olitorius*. Gibberellic acid has greatly

TABLE 1

Effect of Gibberellic Acid on Mean Fibre-cell Length ($\pm$*S.E.*) *for Whole Stem* (*all internodes*) *mm.*

Species	Control	Treated	Increase	% Increase
H. cannabinus	2·80±0·065	3·27±0·102	0·48±0·083	17·1
C. sativa	2·36±0·074	5·57±0·253	3·20±0·164	135·7
C. olitorius	1·82±0·072	2·46±0·116	0·64±0·094	35·3

All differences significant at level $P < 0{\cdot}001$

TABLE 2

Effect of Gibberellic Acid on Mean Length ($\pm$*S.E.*) *of Fibre-cells from 6th Internodes mm.*

Species	Control	Treated	Increase	% Increase
H. cannabinus	2·47±0·051	3·57±0·107	1·10±0·079	44·7
C. sativa	2·63±0·052	6·99±0·186	4·35±0·119	165·4
C. olitorius	1·33±0·025	2·94±0·125	1·61±0·075	121·3

All differences significant at level $P < 0{\cdot}001$

TABLE 3

Effect of Gibberellic Acid on the Percentage Increase in Mean Length of Internodes and Fibre-cells

	6th Internode		Whole Stem	
	% increase in mean fibre-cell length	% increase in mean internode length	% increase in mean fibre-cell length	% increase in mean internode length
H. cannabinus	44·7	113·3	17·1	59·9
C. sativa	165·4	261·4	135·7	185·4
C. olitorius	121·3	202·5	35·3	101·7

increased the scatter of the distribution as well as the mean. It also increases the positive skewness of the distribution of the values of the variate.

The measurements obtained from the thirteenth internodes are expressed in Fig. 2, where the range of fibre-cell length is given together with the mean and standard deviation for control and treated plants of three genera. There is much more variation in fibre-cell length in plants treated with gibberellic acid. It increases the range of cell-length as well as the mean.

If the difference in length is expressed as a percentage increase, it approximates to half the percentage increase in mean internode length induced by gibberellic acid treatment (Table 3). Changes in the dimensions of the

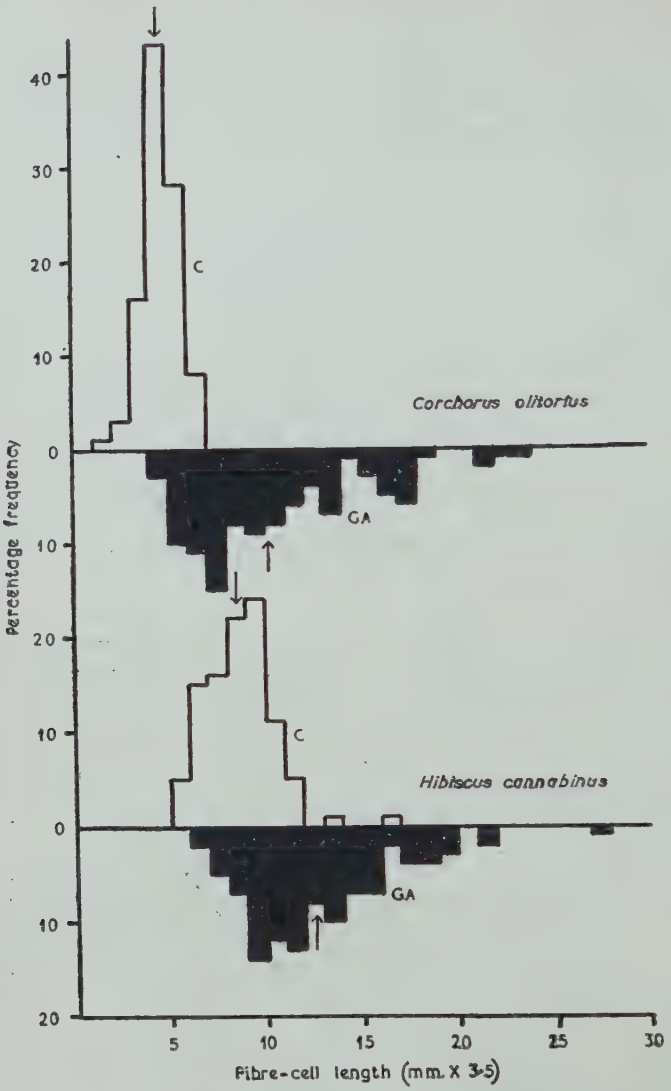

FIG. 1. The frequency distribution of lengths of fibre-cells from the sixth internodes of *Corchorus olitorius* and *Hibiscus cannabinus*. Control, unshaded (C); treated with gibberellic acid, solid black (GA). Arrows indicate the means. Each histogram is based on 100 cells.

morphological components of plants observed during these experiments will be described elsewhere.

A comparison of Tables 1 and 2 shows immediately that the increase in mean fibre-cell length is greater in the sixth internode than for the whole stem. This suggests that there is some variation in fibre-cell length along the stem. Table 4 gives for *Corchorus olitorius* the mean length of fibre-cells for

each internode numbered in developmental series according to the acropetal order of inception. Internode number 1 is the most basal internode at soil level. Internode number 25 is a late-formed internode near the shoot tip. Fibre-cells, or their initials, from the unextended zone below the apex were not measured since the treatment would not produce any effects at this early

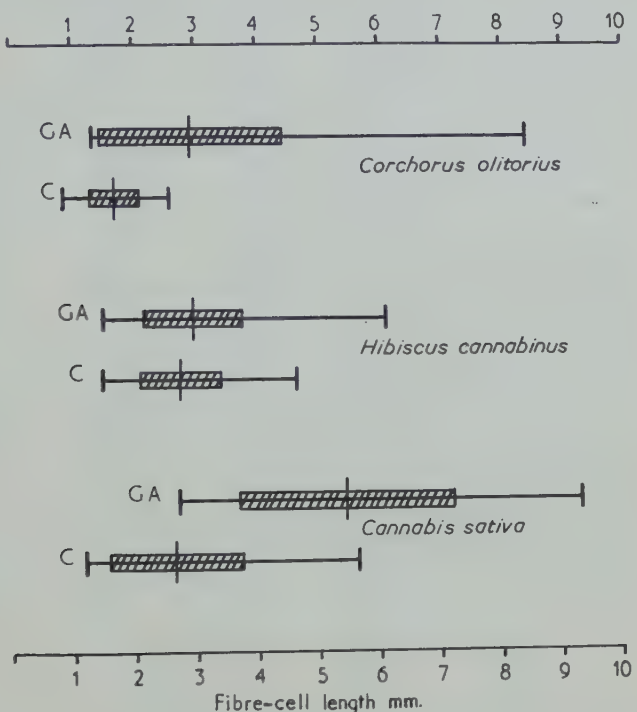

FIG. 2. The range in length of 100 fibre-cells from the thirteenth internodes of treated (GA) and control (C) plants of 3 species. The vertical line near the centre represents the mean length and the shaded portion each side of the mean is the standard deviation.

stage of differentiation. The upper end of the series was also limited by the number of internodes formed by the control plants. Treated plants would regularly produce at least five more internodes from which fibre-cells could be obtained for measurements, but there were no control equivalents for comparative purposes.

The results in Tables 4 and 5 show that treatment with gibberellic acid has produced a highly significant increase in fibre-cell length which is greatest in the basal two-thirds of the stem where the internodes have become fully extended during the course of treatment. At the beginning of the experiment, the first internode was already too mature for its tissues to react to a treatment which affects longitudinal extension.

Fig. 3 illustrates the results given in Table 4. In all except three terminal

TABLE 4

Effect of Gibberellic Acid on the Mean Length ($\pm S.E.$) *of Fibre-cells from all Internodes of* Corchorus olitorius. *mm.*

Internode number	Control	Treated with GA	Increase (difference)	Increase as % of control	Least significant difference at level $P = 0.05$
1	1·07±0·025	1·23±0·023	0·163	14·9	0·23
2	1·15±0·026	1·63±0·048	0·480*	41·2	0·11
3	1·25±0·031	1·92±0·066	0·677*	53·4	0·15
4	1·29±0·029	2·79±0·146	1·501*	116·02	0·30
5	1·38±0·036	2·06±0·053	0·688*	49·2	0·13
6	1·33±0·025	2·94±0·125	1·614*	121·3	0·26
7	2·06±0·111	2·31±0·128	0·256	11·9	0·34
8	1·48±0·031	2·08±0·097	0·604*	41·0	0·20
9	1·59±0·054	2·38±0·100	0·781*	49·1	0·23
10	1·84±0·073	2·90±0·135	1·075*	58·2	0·29
11	1·34±0·035	1·92±0·081	0·583*	43·4	0·18
12	1·67±0·039	1·78±0·062	0·116	6·7	0·45
13	1·69±0·044	2·95±0·156	1·250*	74·0	0·33
14	1·62±0·036	2·45±0·080	0·833*	50·9	0·18
15	1·54±0·024	2·76±0·085	1·224*	79·4	0·18
16	2·26±0·097	3·52±0·138	1·275*	56·4	0·34
17	2·30±0·106	3·00±0·155	0·709*	20·8	0·38
18	3·37±0·102	4·00±0·150	0·637*	18·9	0·37
19	1·76±0·057	1·85±0·098	0·084	5·0	0·23
20	2·61±0·076	2·91±0·096	0·297	11·3	0·24
21	2·43±0·071	3·01±0·146	0·570*	23·6	0·33
22	2·31±0·072	3·02±0·131	0·718*	30·7	0·29
23	2·04±0·392	2·20±0·395	0·166	7·9	0·32
24	2·39±0·109	2·43±0·052	0·039	1·4	0·19
25	1·04±0·025	1·37±0·045	0·320*	31·4	0·10

* Differences significant at level $P < 0.001$

TABLE 5

Effect of Gibberellic Acid on Mean Length ($\pm S.E.$) *of Fibre-cells from Numbered Internodes of* Hibiscus cannabinus *and* Cannabis sativa. *mm.*

Internode number	Control	Treated with GA	Increase (difference)	Increase as % of control	Least significant difference at level $P = 0.05$
H. cannabinus					
6	2·47±0·051	3·57±0·107	1·10*	44·7	0·25
13	2·75±0·066	2·95±0·081	0·20	7·2	0·21
25	3·17±0·077	3·30±0·119	0·13	4·3	0·28
C. sativa					
2	0·88±0·031	1·36±0·037	0·48*	55·0	0·10
3	1·62±0·040	4·72±0·192	3·10*	190·7	0·56
4	1·80±0·034	9·45±0·853	7·65*	424·7	0·80
6	2·63±0·052	6·99±0·186	4·36*	165·4	0·70
8	3·93±0·128	5·48±0·160	1·55*	39·4	0·41
11	3·02±0·117	5·50±0·166	2·48*	82·4	0·55
13	2·64±0·114	5·46±0·178	2·81*	106·3	0·47

* All differences significant at level $P < 0.001$

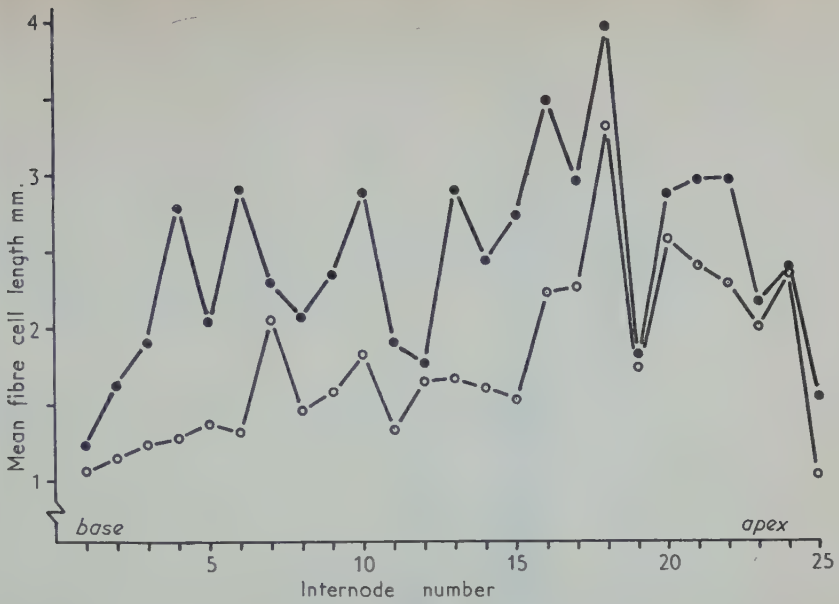

FIG. 3. The variation in fibre-cell length with internode sequence and the effect of gibberellic-acid treatment in *Corchorus olitorius*. o—o, control; ●—●, treated with gibberellic acid.

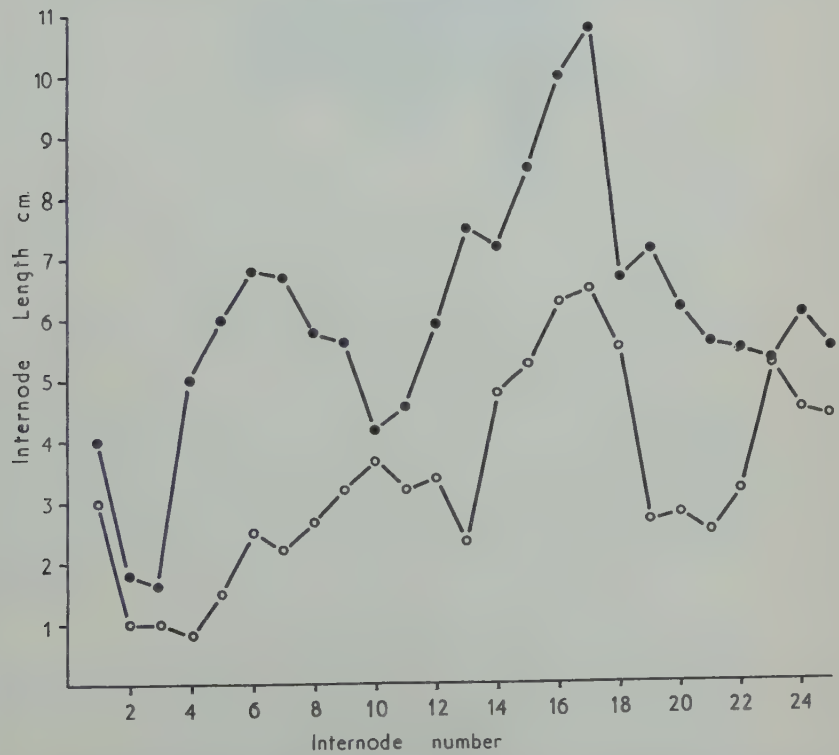

FIG. 4. The variation in internode length with sequence of development and the effect of gibberellic-acid treatment in *Corchorus olitorius*. o—o, control; ●—●, treated with gibberellic acid.

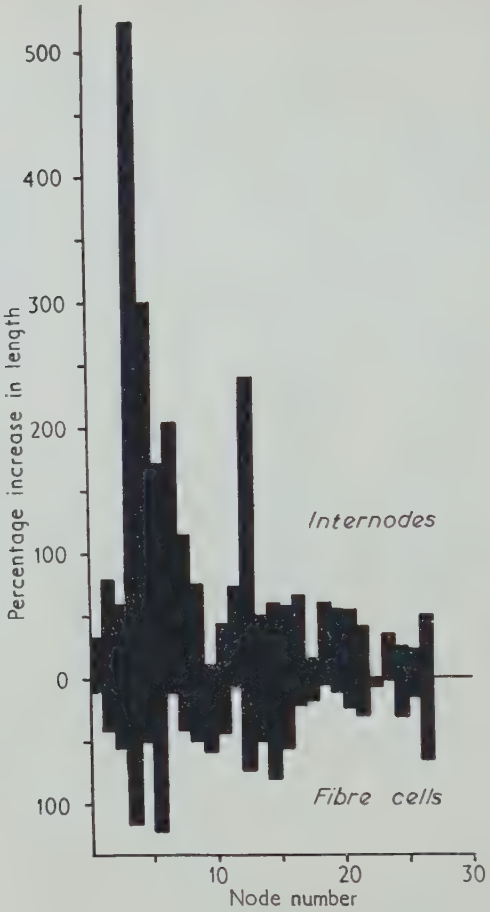

FIG. 5. Increase in length of internodes and fibre-cells of *Corchorus olitorius* induced by treatment with gibberellic acid, expressed as percentage of control.

internodes, the mean fibre-cell length of treated plants is higher than the average of the means for all internodes of controls, the exceptional internodes being terminal. Although there are often considerable differences in mean fibre-cell length between adjacent internodes, in the untreated plants there is a gradual increase up the stem, reaching a maximum about the eighteenth internode, followed by a more rapid decrease towards the apex. The pattern is repeated in treated plants but in a more exaggerated form. The increase is more sudden in the lower first-formed internodes and reaches a fairly steady level apart from a maximum peak, again at the eighteenth internode. In effect, gibberellic acid accelerates development by two or three plastochrones, the primary peak in fibre-cell length, and that equal to the maximum occurring in control plants, being reached about three internodes lower down the stem in treated plants.

Variations in internode length show a similar developmental sequence (Fig. 4), with gibberellic acid inducing increases in length according to the same pattern as the effect on fibre-cell length. In Fig. 5 the percentage increase in fibre-cell length and internode length are compared for all internodes. Both units show a similar reaction pattern to the growth stimulus, though there is a quantitative difference of two or three times in the amounts of elongation induced. The morphological unit lengthens proportionately more than the histological one.

Discussion

Gibberellic acid produced highly significant increases in fibre-cell length at all levels of the stem and in all the plants examined. This increase was not constant throughout development but exhibited a definite pattern which can be related to the variation in internode length, a unit which shows a similar reaction to the application of gibberellic acid. This substance increases growth, and it also accelerates and extends it. The maximum cell length and internode length are reached at an earlier stage developmentally, and the plastochrone interval is shortened, so that there may be 2–5 additional internodes on treated plants from which elongated fibre-cells can be obtained.

It would not be appropriate for the author of this paper to attempt any evaluation of possible economic applications of this work, though such postulations have been latent throughout the experiment. Gibberellic acid induced the production of much longer fibre-cells and also increased the number of internodes from which they are available. It was observed that cells from treated plants seemed more liable to break during manipulation. It is possible that these cells were more brittle, or the explanation may be that their greater length rendered them more difficult to retain complete. It would be interesting to know whether gibberellic acid has affected the physical or chemical structure of the cell-wall and its strength.

From the practical point of view it was noticed that when groups or 'bundles' of fibre-cells were carefully pulled out from the mass of fibrous tissues after retting and before maceration, these were regularly as many as four times longer in treated plants than in controls. Some measurements of this fact were made, and it was found to be constant and statistically significant. These results have not been given in detail, since they are not of scientific value. They are, however, mentioned here, since, commercially, fibres consist of such groups of individual fibre-cells.

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We are indebted to Plant Protection Ltd. for their generous provision of gibberellic acid.

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Xylem Occlusions in the Fruit of Cacao (*Theobroma cacao*) and their Relation to Cherelle Wilt

BY

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With one Plate and two Figures in the Text

Abstract

The anatomy and distribution of the xylem of the fruit of cacao (*Theobroma cacao*) has been investigated by injection and staining techniques.

The physical symptoms of cherelle wilt (pod wilt), a physiological fruit-thinning mechanism, have been shown to be caused by occlusions in the xylem vessels of the fruit-stalk. The initiation of these occlusions is thought to be associated with the oxidative activity of a cambial or meristematic band of tissue, which forms new vascular tissue to the pericarp.

Introduction

CHERELLE wilt of cacao is the shrivelling and blackening of young cacao pods, a physiological disorder which may account for a considerable loss of the potential crop. The percentage of pods lost by wilt is very variable and the figures of Pyke (1933) illustrate the variation between trees, from 19 per cent. on some trees to 92 per cent. on others. Humphries (1943*a*) has shown that a major contributory factor in cherelle wilt is the competition for nutrients and water not only between individual fruits but also between the fruits and new vegetative growth. From his data, Humphries has inferred that cherelle wilt is analogous to the fruit-thinning mechanism of other trees. There is no evidence in the extensive literature on the subject that this interpretation is incorrect and therefore cherelle wilt is considered to be a physiological fruit-thinning mechanism, as much part of the overall growth phenomena of cacao as 'flushing', the rapid growth of vegetative buds, or leaf abscission. It is important to realize that the fruits of cacao do not absciss, unlike many tropical and temperate fruit trees, but merely shrivel and dry until they are displaced from the tree by external causes. Cherelle wilt must play an important part in the determination of the magnitude of the final crop and it is therefore important to understand the underlying causes which contribute to its expression.

In a consideration of the factors which contribute towards cherelle wilt, two phenomena must be involved. Firstly, there is the mechanism by which the physical symptoms of wilt are expressed, i.e. the immediate cause of the shrivelling of the pod, and secondly, the contributory factors which instigate this mechanism. An analogy may be drawn to other trees such as the apple,

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in which the development of the abscission layer of the peduncle of the apple fruit may be considered the 'mechanism' of fruit drop, whereas nutrients, auxin, water, &c., are part of the contributory factors. With regard to cacao, the attention of previous workers has been confined to the contributory factors of cherelle wilt and not to its actual mechanism. The factors which have been claimed to be contributory to wilt are many. Humphries (1943*a*) claimed that wilt was due primarily to nutrient and water deficiency and Pound (1932) showed that weather was an important factor. Miller (1954) claimed that mechanical damage may accentuate wilting. McLaughlin (1950) inferred from the results of spraying trials with Bordeaux mixture that fungal infection by *Phytophthora palmivora* (black pod) is a contributory feature of cherelle wilt, although the symptoms of these diseases are difficult to distinguish from each other. Most workers have claimed that, although wilted pods are heavily infected with many organisms, they are secondary invaders entering the pods after wilt has started. From the observation that wilting occurs in waves, McKelvie (1956) has inferred that endogenous auxins of the pod may be involved as well as the nutrient status of the tree; Nichols (1959), from measurements of auxins in the seeds, has confirmed this view.

Nevertheless, the contributory factors are not separate entities and are clearly interrelated, e.g. climate cannot be separated from water-supply, nutrient status, and so on. It is the object of this paper to present a hypothesis which may account for the initiation and physical expression of wilt.

There are two pointers which indicate that the mechanism of wilt may be associated with vascular bundle organization:

(i) the cessation of growth of a pod a few days before physical symptoms of wilt appear,

(ii) after 80–100 days from fertilization, when the pod has reached an approximate length of 10 cm., wilt rarely occurs; the pod may be lost from other causes but not from true physiological wilt.

The inference from a consideration of both of these factors is that either cambial growth or vascular differentiation may be affected, which in turn causes the physical symptoms of wilt.

Methods

The anatomy of the cacao pod has been investigated in two ways. The gross distribution of vascular bundles has been examined by injection of pods through the pod stalk with basic fuchsin, and the histology by saffranin staining. Each method will be described in the appropriate section.

Injection of pods

Basic fuchsin (0·05 per cent.) dissolved in 2 per cent. ethyl alcohol was pipetted into a series of U-tubes one arm of which was attached to a manifold and connected through a gas-reducing valve to a cylinder of nitrogen. Healthy pods were freshly severed from the tree and a fresh cut made in the pod stalk before insertion into a short length of pressure tubing attached to the free

end of the U-tube. Sufficient basic fuchsin was pipetted into the pressure tubing for the displacement of liquid by the pod stalk on insertion to expel any entrained air bubbles. A light smear of lanolin was used to ensure an adequate seal between the epidermis of the pod stalk and the pressure tubing. Whenever small pods 4–5 cm. in length were examined, they were mounted in a similar fashion, but the seal was implemented with a short sleeve of bicycle-valve rubber, sheathed over the pod stalk. After insertion in the U-tubes, the reducing valve on the nitrogen gas cylinder was opened slowly until a pressure of 5 lb. per sq. in. was recorded. The gas pressure was maintained without further adjustment for varying periods depending on the size of the pod, 5–7 hours usually being sufficient for a general staining of the xylem tissue, or until a removal of a slice of tissue at the distal end of the pod revealed that the xylem elements had been adequately stained.

Healthy pods

After the pods had been injected they were examined macroscopically by cutting serial cross-sections with a razor blade.

Distribution of vascular tissue

The broad picture of the vascular tissue revealed is that the pod is served by three discrete longitudinal systems.

The first system consists of five central bundles, which run virtually the whole length of the pod and are continuous with the vascular system of the peduncle. The xylem structure of the peduncle is similar to that of the stem, which has been described by Brooks and Guard (1951). The pith in young pod stalks is distinctly parenchymatous but the cells may break down in older tissues and form a mucilaginous cavity. The secondary xylem is composed of tracheids and vessels and is traversed radially by medullary rays. Owing to overgrowth of the pod wall at its proximal end, the peduncle becomes partially enveloped in the pod wall. The second system, lying in the parenchymatous ground tissue, consists of five bundles, radial to the five central bundles and external to the seeds.

The third system consists of a band of bundles, distributed along the inside of a pentagonal tissue which is clearly visible in the young pod. At the five corners of the pentagon the bundles are larger than those along the inside edges of the pentagon. The interesting feature, however, is the large number of small bundles which are found along the inside of the pentagonal tissue. This tissue is thought to be a band of highly meristematic cells, which not only initiates bundles *de novo* but may also be responsible for some part of the increase in the girth and length of the pod; this point is discussed more fully later in the text: Fig. 1 diagrammatically illustrates the distribution of the vascular tissue through a pod 8 cm. in length.

A further interesting feature is that although the pod wall appears to be heavily stained with fuchsin after injection, the bundles which are responsible

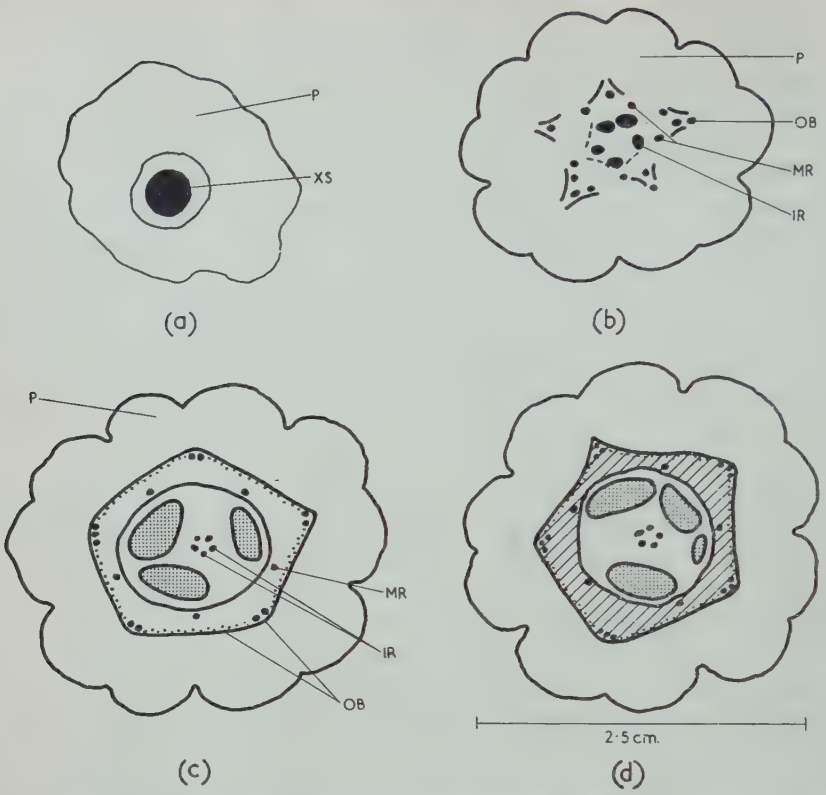

FIG. 1. Healthy pod, 8 cm. long, injected with basic fuchsin, showing vascular bundles at different levels in the same pod. Black dots = vascular bundles; dotted areas = seeds cut at different levels; cross-hatched areas in (*d*) = area of rapid oxidative browning in a damaged, healthy pod, or area of precocious oxidation in wilting pods.

The numbers and distribution of vascular bundles at the corners and sides of the pentagon are not to scale; lateral bundles not shown. P = pericarp; IR = inner ring of bundles; MR = middle ring of bundles; OB = outer band of bundles; XS = xylem of stalk.

(*a*)–(*d*). Transverse sections of pod at levels indicated:

(*a*) Point of insertion of stalk into pod.
(*b*) 0·5 cm. from (*a*).
(*c*) 2·0 cm. from (*a*).
(*d*) 4·0 cm. from (*a*), i.e. median transverse section.

for this appearance do not form an organized system, but are scattered throughout the wall and they all appear to link with the outer band of vascular bundles. This conclusion has been established by cutting serial sections through the pod wall and tracing individual bundles in the wall until they appear in longitudinal section; they all appear to emanate from the outer ring of vascular tissue.

The overall picture of the distribution of the vascular bundles in the healthy pod appears to consist of three distinct systems, each connected by lateral or medullary rays. The systems and the tissues which they serve are summarized below.

(i) An inner ring of five bundles associated with the seeds and the ground tissue surrounding them.

(ii) A middle ring of five bundles radial to the inner ring, serving the ground tissue external to the seeds.

(iii) An outer or pentagonal band, with five large bundles in each corner of the pentagon and numerous small bundles along the five sides; this system serves the ground tissue of the pod wall.

Zamora *et al.* (1960), during investigations on the ontogeny and embryology of the cacao flower, have illustrated the vascular traces of the ovary before fertilization. It seems clear that the 'ventral' traces of the ovary in their description are homologous with the inner ring of bundles described above. The largest of the traces which they describe as lateral traces, radial to the inner ring, probably form the middle ring of bundles, as a result of differential growth after fertilization. Their 'dorsal' traces form either the first bundles of the pericarp, which are then superseded by the pentagonal band, or become part of it.

Healthy and wilting pods

In order to compare the vascular organization of the healthy pods with that of wilting pods, similar injection experiments were conducted with pods showing various stages of wilt. The first experiments were conducted with pods of different ages which showed obvious symptoms of wilt, viz. premature yellowing. However, using this type of pod, little or no dye could be injected at all, which suggests that at least one factor responsible for wilt was either a blockage, or a lack of differentiation of the vascular system, either in the pod or the pod stalk. In order to differentiate between pod and stalk, stalks severed from wilting and healthy pods were mounted for injection on the apparatus described and treated similarly with fuchsin. The results, although not recorded on a quantitative basis, were identical with those of the experiments with pods and stalks combined, i.e. the stalks from wilted pods would not permit passage of the fuchsin, whereas the stalks of healthy pods were adequately stained and permitted free flow of the dye. Clearly, an obstructive mechanism was present in the pod stalks and possibly in the fruits too. No polarity effect was involved because identical results were obtained with either the proximal end or the distal end of the stalk mounted in the manifold.

However, once the pods express visible symptoms (late wilt) many of the observed characteristics may be entirely secondary, and therefore it was necessary to examine pods before the appearance of late wilt symptoms. The fact that pods about to wilt cease elongation shortly before showing visible symptoms was used (Cheesman, 1927).

Fifty cherelles were chosen at random on trees of I.C.S. 1, numbered, and increments in length noted at the same time daily. Representative examples showing the variation between individual pods is shown in Fig. 2. Although it is quite clear that in the wilting pod after the increase in length has ceased there is an approximately 4- to 6-day period before a decrease in length is

apparent, there is considerable variation between pods and in some cases healthy pods may apparently cease growth for 2 days and then resume growth. Whether this is due to unavoidable error in measurement, or whether it represents a true picture of the growth pattern, has not been determined. Therefore, in selecting material for microscopical or subsequent injection

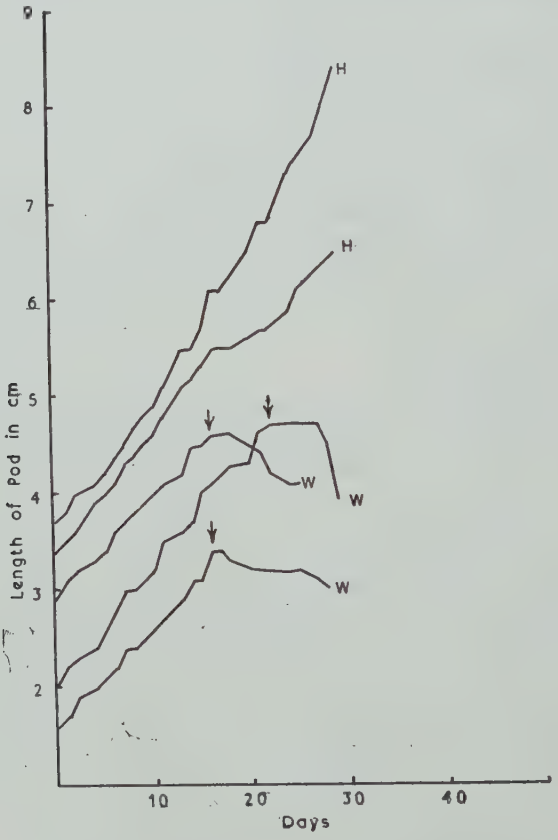

FIG. 2. Increase in length of two pods (H) which remain healthy and three which wilt (W). The inverted arrow indicates the point of incipient wilt.

examination, pods have been described as being in early wilt when they show not only a cessation in growth for 2 days but also a slight loss of bloom. No pods which have satisfied this criterion have been shown to remain healthy. Incipient wilt is used to describe the time at which an increase in length of the pod was last recorded. Table 1 shows the uptake of fuchsin on a semi-quantitative basis observed in the pods at various times from incipient wilt.

The inference from the injection experiments using selected pods, that is pods of approximately known age from the commencement of incipient wilt, is that there is some obstruction to liquid flow in the pod or stalk. Furthermore, the occlusions or blockages increase with time from incipient wilt

onwards. From the results of the injection experiments with pod stalks, it seemed likely that the stalks themselves were in some way involved and probably the xylem in particular.

TABLE 1

Relationship between Degree of Staining and Age of Pod from Incipient Wilt

Days from incipient wilt	Length of pod (cm.)	Degree of staining	Visual symptoms of wilt
(Healthy) —	3·7	+++	Nil
(Healthy) —	4·2	+++	Nil
2	3·2	++	Loss of bloom
2	3·8	++	Loss of bloom
3	3·1	+	Slight yellowing
4	4·6	+	Slight yellowing
6	2·8	o	Slight blackening
6	2·4	o	Slight blackening

o = no stain in vascular tissue of the pod.
\+ = slight stain in vascular tissue of the pod but not in wall.
++ = slight stain in vascular tissue of the pod and slight in wall.
+++ = heavy staining in the vascular tissue of the pod including wall.

Anatomy of the Xylem of Stalks from Wilting and Healthy Pods

The blockage in the pod stalks was investigated by cutting serial sections of prepared material at 25μ on a microtome. The fixative used in all cases was alcohol-formalin-acetic acid and the sections were stained with saffranin only.

Figs. 3 and 4 in the Plate show transverse sections of the pod stalks from a healthy and a *late* wilting pod, both of comparable age; the darkly stained occlusions in the vessels of 4 can be clearly seen. All stalks from pods at the early wilt stage and later, which have been examined in this fashion, have shown vessel occlusions.

The essential difference between the occlusions in the early and late wilting stages is one of quantity. In the late wilt stalks virtually all the vessels show the occlusions, whereas in the early wilt phase a number of the vessels remain clear.

The nature of these vessel occlusions has been examined and the evidence suggests that they are not tyloses but mucilage plugs for the following reasons.

(i) Examination of Fig. 4 shows that the occlusions are angular, broadly following the outline of the parent vessel. This may be expected if the mucilage plug, which had originally filled the vessel, had contracted by dehydration when transferred through the alcohol series during fixation. A tylose may be expected to contract irregularly under similar treatment, unless the middle lamella from which it had been derived had been subsequently thickened. No evidence for such secondary thickening has been observed.

(ii) In transverse sections extrusion of the middle lamella and cell contents from adjacent xylem parenchyma through pits has not been observed.

(iii) In unstained sections of fresh material the occlusions appear as translucent amorphous bodies, either completely filling the vessel lumen or

dispersed irregularly across it. The occlusions from fresh material stain faintly with saffranin. Mucilage when expressed from cacao-pod wall tissue, partially dehydrated with alcohol and stained with saffranin, gives an appearance similar to the occlusions in the preserved specimens.

(iv) If the occlusions were tyloses, formed by a difference in hydrostatic pressure between the vessel and an adjacent xylem parenchyma cell, initiated by some unspecified system peculiar to a wilting pod, then healthy pods which had been allowed to dry naturally would also be expected to show tylose formation. In order to test this hypothesis, 9 pods of approximately similar length were selected at random from one tree and allowed to dry on a laboratory bench. The pod stalks were examined by fresh and fixed sections on successive days for a 4-day period, sufficiently long for a wilting pod on the tree to show occlusions in the vessels; no comparable occlusions were observed.

From a consideration of the factors i–iv, there appears to be little doubt that the occlusions are not tyloses; on the other hand, the staining and general appearance suggest that they are probably mucilaginous in origin. However, the progressive development of the occlusions indicates that they are not primarily responsible for the initiation of wilt, but are themselves secondary responses, responsible for the external symptoms of the wilting phenomena and in particular the late wilting phase. It is reasonable to infer that the true initiation of wilt will be found in the pod itself.

The cacao fruit is described as a berry containing from 20 to 40 seeds depending on the variety. The source of material used in these investigations was a Trinitario-type cacao, Imperial College Selection 1 (I.C.S. 1), a clone which bears yellow-red pods at maturity. The mature pod is ovoid and may be as long as 20–25 cm. in length and 10 cm. in diameter. The fleshy pericarp, approximately 1·0 cm. in thickness, consists of a thin pigmented epidermis and hypodermis, surrounding a parenchymatous cortex which contains numerous irregularly dispersed mucilage cavities. A narrow band of woody tissue is located within the cortex about two-thirds from the epidermis. The seeds, the 'beans' of commerce, are attached to the central axis of the pod as a tightly adhering mass. At maturity the seeds are surrounded by a pulp developed from the outer integument of the ovule; at full ripeness, as a result of some dehydration of the pulp, the seed-mass becomes separated from the pericarp and lies loosely within it. The description which follows of the anatomy of the pod is concerned only with pods smaller than 10 cm.; it has been mentioned that pods rarely wilt after attaining this length.

The gross external features of a wilting pod are quite obviously different from a healthy pod, but on internal examination the obvious difference is in the degree of browning of the pentagonal tissue, corresponding to the cross-hatched area in Fig. 1*d*. In early- and late-wilting pods of all ages this area is heavily oxidized without having been damaged by breakage, although in healthy pods this area becomes heavily oxidized within 1–1½ minutes after cutting open; on immediate examination after cutting the area is creamy

white. It was because of the apparent association of the oxidation of the cell contents of this layer with pod wilt that the anatomy of it and the associated tissues were investigated.

Brief mention has been made of the outer pentagonal band of vascular bundles which serve, by lateral rays, the tissues of the pod wall. This band of bundles is adjacent on its outer side to a tissue which is thought to be a cambium. The area of precocious oxidation in the wilting pods corresponds to this cambium, the band of vascular bundles, and the inner tissues of the

TABLE 2

Relationship between Length of Pod and Number and Dimensions of Vascular Bundles in the Outer Ring

Estimated age days	Pod		*Approx. number of bundles per millimetre	†Approx. range in size of bundles μ	‡Approx. distance of bundles from cambium μ	
	Length cm.	Diam. cm.			smallest	largest
20	2	0·7	10	20–40	20	50
30	3	0·9	8	20–50	20	50
40	4	1·3	9	20–90	20	100
45	5	1·7	8	20–50	20	100
55	7	2·2	8	20–140	20	100

* Measured along one face of the pentagonal cambium.
† 20μ is the smallest size of bundle at which individual elements are discernible.
‡ Measured from estimated centre of vascular bundle to internal edge of cambium.

pericarp. Clearly, if this band of bundles is derived from the cambium then its activity, or lack of activity, may well account for the cessation in growth of a pod at incipient wilt. Because it appears to be unusual to find an organized cambium in fruits, evidence will be presented that it does in fact produce the outer band of bundles.

Healthy pods 2, 3, 4, 5, and 7 cm. in length were chosen for investigation. They were first cut traversely half-way along their length and a suitable segment was removed from the basal portion in preparation for microtome sectioning. The pod segments were fixed in alcohol-formalin-acetic acid solution, sectioned at 15μ, and the sections, after staining in either haematoxylin or safranin, were examined.

The first observation was that the outer ring of the bundles consists of a large number of discrete vascular bundles of varying sizes forming a band around the inside of the cambium. The increase in size, with the smallest adjacent to the cambium and the largest centripetally, suggests that these bundles arise from the cambium and not from the surrounding ground tissue. Table 2 shows the relative dimensions of the vascular bundles underlying the cambium tissue. At approximately 20μ the bundles are just discernible as discrete entities, although smaller groups of cells which probably differentiate into bundles may be seen close to the cambium. The relative constancy of bundles per millimetre confirms the view that the bundles arise *de novo* from

the cambium and that they are not primarily laid down earlier in the ontogeny of the fruit. It must be inferred that the parenchymatous tissue internal to this outer band of bundles is also actively meristematic because the bundles do not become distributed within the ground tissue as the pod grows, but maintain their proximity to the cambial tissue.

Examination of the meristematic tissues in question, stained with haematoxylin, has not revealed mitotic figures, but the nuclei are enlarged in the cambial tissue and in the adjacent parenchymatous tissues. However, in cross-sections of ovaries stained with haematoxylin, taken from hand-pollinated flowers 1, 2, and 3 days from fertilization, mitotic figures may be clearly seen in a band of tissue between the seeds and the epidermis. This band of tissue (perhaps better described in this case as a 'meristematic ring') is more nearly circular at this stage and is composed of small cells with enlarged nuclei and it is from this layer that the pentagonal cambium of the older pod is formed. Even within the 3-day-old pod, the meristematic ring appears to differentiate centripetally individual groups of cells which presumably form the first vascular bundles of this layer.

The development of lateral rays from the cambium to the ground tissues of the pod wall can be clearly seen in the Plate, Fig. 5. The progressive development of these lateral rays appears to be a feature of the development of the fruit, because although lateral rays may be seen in younger fruits, they occur with a lower frequency than in the older ones. In older fruits, nearing maturation, these rays can be seen macroscopically simply by splitting the pod lengthways.

Except for a marked increase in the number of heavily stained cells in the cambium and adjacent tissues and a general appearance of loss of turgor within the cells, the gross anatomy of wilting pods appears to be similar to that of healthy pods; the heavily stained cells contain the brown oxidation products of naturally occurring polyphenols.

Culture of tissue explants

Preliminary experiments on the activity of the tissues taken from the region of the outer band of vascular bundles and cambium, using tissue isolates from both healthy and wilting cherelles, have been conducted. The technique was adapted from White (1943). Blocks of tissue from the cambial regions of surface-sterilized pods (5·5 cm. in length) at the healthy and early wilting stage were grown aseptically on agar, containing White's medium. Within 48 hours the cambial region of the healthy pod had enlarged visibly although growth subsequently ceased after 7 days. As was expected, no comparable growth was observed from the oxidized cambial tissues isolated from the wilting pod. However, subsequent callus-like growth from the tissues internal to the cambium, corresponding to the inner meristematic tissue of the pericarp, has been observed from both types of explant. These preliminary results indicate that, at least in the early stages of wilt, general necrosis of the pod tissue is not apparent.

Discussion

Evidence has been presented that the wilting of young fruits of cacao is caused by a blockage of the xylem of the fruit-stalk, caused by occlusions within the lumens of the vessels. However, it is considered that the xylem blockage is a secondary feature of the wilting phenomena and that it is not the primary cause of wilt, because the occlusions develop progressively, following the cessation of pod growth at incipient wilt. The more plausible explanation is that there are two distinct phases of the wilting phenomenon:

(i) a cessation in growth of the cambium of the fruit, followed by
(ii) development of the vessel occlusions.

The evidence presented that the cambium is involved is inferential, but the following arguments are compatible with this view. Healthy and wilting pods differ internally by the appearance of the oxidized band in the wilting pod before mechanical disruption of cells, e.g. on opening, has occurred. Within the healthy pod the oxidation is exhibited only after mechanical damage has occurred, i.e. after the opening of the pod. Clearly, in the wilting pod, spatial separation of the enzyme (polyphenol oxidase) and its substrates has broken down and oxidation of the substrate has proceeded. The area of active internal oxidation in the wilting pod has been shown to be associated with a cambial or meristematic band of cells, or at all events a tissue responsible for the formation of new vascular tissue. As the pod develops in size, lateral rays develop within the pericarp from the cambium and link with the new bundles formed from the cambium. It may be inferred that if the cambium ceases to grow, further vascular development will also cease. The first symptoms of wilt are a slight loss of turgor and a cessation in elongation of the pod for a few days. These observations support the view that *immediate* blockage of the vascular tissue does not take place either in the pod wall or in the pod stalk at incipient wilt; an immediate rapid shrinkage of the pod would be expected if either of these events did, in fact, occur. It appears then, that for the first 3 or 4 days from incipient wilt, an approximate equilibrium is maintained between water loss from the pod wall and water transport to it. However, during this period the vascular occlusions are forming in the pod stalk, slowly imposing a restriction in water-supply to the pod as a whole, until complete, or nearly complete, occlusion of the vascular tissue occurs and rapid drying of the pod ensues.

It must be stressed here that the vascular obstructions described in the text have been observed only in the vessels of the pod stalks and never in the tracheids, therefore the term 'vascular' occlusion must be accepted with some reservation. It may be true that the tracheids are similarly blocked, but that the responsible precursor is not present in sufficient quantity to be apparent by the techniques used in this study. Indirect evidence has been presented in the text that the occlusions in the vessels are mucilaginous; even so it cannot be excluded that the occlusions may be oxidation products of polyphenols. Wilting of tomato plants infected with *Fusarium oxysporum*

f. lycopersici has been ascribed to blockages in the xylem vessels, caused at least in part by oxidation of mobile phenols which form quinones or melanins in response to the host enzymes (Waggoner and Dimond, 1955). However, owing to the general occurrence of mucilage receptacles in cacao, it is reasonable to assume, in the absence of evidence to the contrary, that the blockages are of mucilaginous origin.

The overall sequence of events which is envisaged to take place within a pod which wilts is as follows:

Lack of auxins and/or nutrients
↓
cessation in growth of cambium
↓
oxidative browning of cambium and parenchymatous ground tissue
↓
loss of turgor (*symptoms of early wilt*)
↓
transport of stimulus (phenols or quinones?) within vascular system
↓
occlusions in vessels of pod stalks
↓
restricted water-supply (*symptoms of late wilt*)

The hypothesis concerning the mechanism of wilt is reasonable with respect to the young fruits (<10 cm. in length), although it might be argued that it could be equally applicable to older pods which, however, do not wilt. The temporal difference, used in this sense, refers only to the capacity of the fruits to wilt and therefore the actual size and age differences are relatively unimportant. There are, however, important metabolic and physical changes which go on in the pod approximately 75 days from fertilization when the fruit loses its capacity to wilt (Humphries, 1943*b*). One such change, illustrated by the data of Humphries, is the ratio of fruit diameter to fruit length. At about 80 days the ratio changes from 0·35 to 0·45 at 100 days, i.e. the fruit swells; the ratio subsequently remains constant at this new level. It may be postulated that this change corresponds to the laying down of the final skeleton of the vascular tissue of the fruit wall, that is, once the full development of the vascular tissue of the wall is established the wall becomes independent of the attendant cambium and therefore true physiological wilt may not occur; this point is being investigated further.

Acknowledgements

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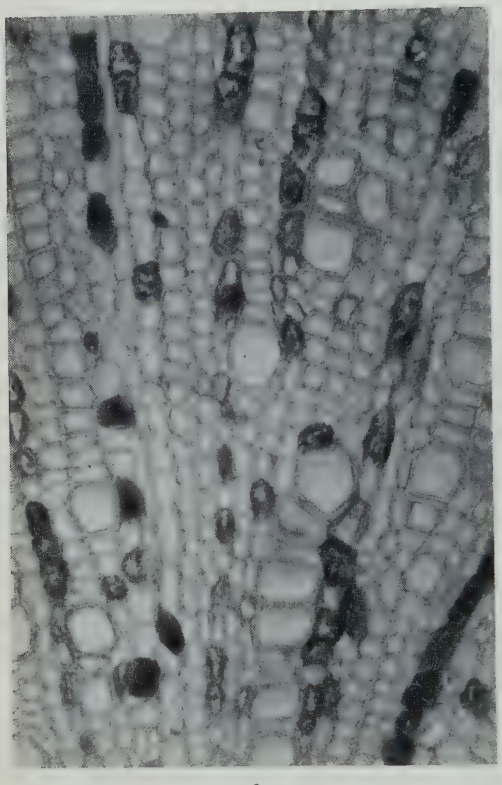

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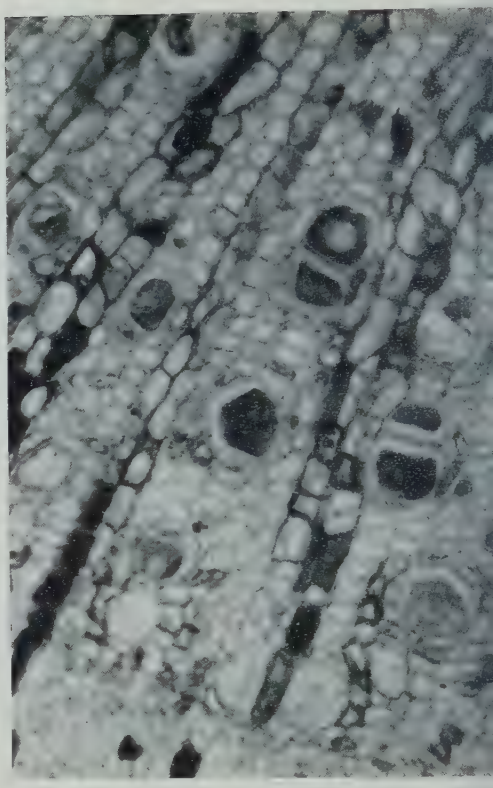

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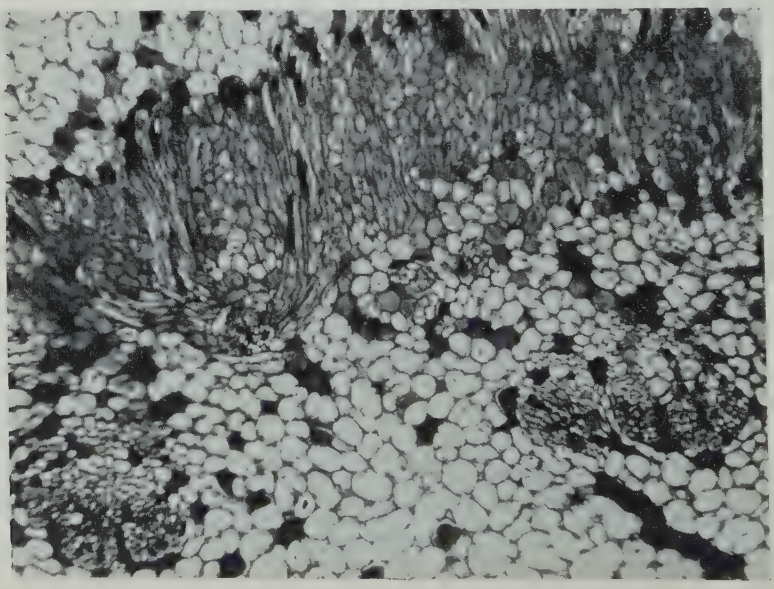

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EXPLANATION OF PLATE

Fig. 3. T.S. of pod stalk of healthy pod, 5·1 cm. long.

Fig. 4. T.S. of pod stalk of late wilting pod, 5·0 cm. long. The darkly stained occlusions can be clearly seen in the vessels. Both sections stained with saffranin. ×288.

Fig. 5. T.S. Pericarp of pod 7 cm. long, through the band of meristematic tissue (darkly stained, *centre left to top right*) with the vascular bundles along the inner (lower in photograph) side. Two large vascular bundles can be seen lower left and right. Smaller bundles may be seen closer to the meristematic tissue, with lateral rays joining one of them (*centre left*). The central axis of the pod is out of the picture, but would be away from the lower right-hand corner.

Stained with haematoxylin. ×288.

Experimental and Analytical Studies of Pteridophytes

XXXIX. Morphogenetic Investigations of Sori in Leptosporangiate Ferns

BY

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With two Plates and twenty-four Figures in the text

ABSTRACT

The fact that there is a demonstrable unity of origin of the meristematic tissues in ferns, and that developments of an adventitious character are rare or absent, has led to the view that the young soral receptacle, or placenta, is a special kind of meristem, now described as a sporogenous meristem. These meristems originate as growth centres in the leaf marginal meristem, the latter being now recognized as an organized formative region sharing many of the properties and functions of the shoot apical meristem. According to the nature of the growth relationships in the marginal meristem after the onset of the reproductive phase, sporogenous meristems may remain in a marginal or intramarginal position, or they may come to occupy a superficial position on the abaxial side of the leaf. In those species in which the further growth of the leaf margin is more or less completely inhibited as sorus development proceeds, the mature sori occupy marginal or intramarginal positions: on the other hand, in species in which marginal growth is sustained, the sori eventually occupy superficial positions on the abaxial leaf surface. Thus the phylogenetic shift of sori from a marginal to a superficial position, as envisaged by evolutionary morphologists of an earlier period, has now been shown to take place in the individual species of *Superficiales* as an ontogenetic development. Information of this kind is considered to be of special value and interest in that it admits of a convergence of the ideas that have emerged from both morphogenetic and phylogenetic studies.

1. INTRODUCTION

THE fern sorus typically originates from a localized area of embryonic tissue, described as a *receptacle* or *placenta*, which may be situated on or within the leaf margin or on the lower surface of the fertile leaf or sporophyll. Goebel (1905) preferred the term placenta to receptacle, but both are in current use. He regarded a placenta as a region of particular importance, with special nutritional relationships, and capable of giving rise to the sorus. Investigators of pteridophytes, including Hofmeister among the earlier workers and, later, during the post-Darwinian period, Goebel, Campbell, Bower, and others, carried out much detailed and meticulous anatomical research on the spore-producing members. Their descriptions and illustrations, indeed, constitute the foundation upon which all further work must be based. Because of their preoccupation with problems of evolution and phylogeny, the contributions of post-Darwinian botanists on the sorus were mainly

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in the sphere of comparative morphology. Causal aspects were not, however, completely overlooked and some interesting observations on related ecological aspects were also made, e.g. in Goebel's *Organography of Plants*. Studies in which morphogenetic aspects are put first are still rather occasional and references to them are virtually lacking in standard texts. A review of this work and of the general inferences which can be drawn from earlier morphological investigations has been given by Wardlaw (1961). A summary of the literature on the development of fern sori has been prepared by Sharma (1959).

The present investigation is based on the simple concept that the soral placenta is *a special kind of meristem*, in fact, a *sporogenous meristem* (Wardlaw, 1958, 1961), and on the view that the whole process of soral inception and development merits re-examination from the morphogenetic standpoint, i.e. along the lines which have already proved fruitful in the study of the meristems of shoots, buds, leaves, roots, and flowers. In the ferns, adventitious organ formation is apparently rare: virtually *all* the embryonic or meristematic regions can be referred to a pre-existent meristematic or embryonic tissue, the shoot apical meristem being specially important. Accordingly, the origin of the sporogenous meristem, the factors which may be involved in its further development to the fully elaborated sorus, and the distinctive and characteristic position which it occupies, are the principal aims of the present investigation.

The regular distribution of sori excludes the possibility of their being adventitious in origin. Nor does it seem probable that they arise as a result of dedifferentiation of mature epidermal and mesophyll tissues. The many published illustrations and the present writers' observations of soral formation in *Dryopteris aristata*, *Phyllitis scolopendrium* (*Scolopendrium vulgare*), *Cryptogramma crispa*, *Athyrium filix-femina*, and *Polystichum aculeatum* indicate that the sporogenous meristem can be directly related to the primary growth and differentiation of the sporophyll. As some of the earlier investigators well understood, leaf formation in ferns, including its shape and 'architecture', is best understood in terms of the activity of its distal and marginal meristems.

If a sorus is formed from a special kind of meristem, it is a reasonable assumption that it originates from a pre-existing meristematic region of the leaf and, moreover, that it contains some particular substance or substances necessary for the formation of sporangia. From the many published accounts it may be inferred that sporogenous meristems typically originate in the marginal meristems. Indeed, Wardlaw (1961) has suggested that, subject to some qualification, all fern sporogenous meristems originate in this way; and he has tried to explain the various positions (marginal, sub-marginal (intramarginal), superficial and others) occupied by the mature sori, in terms of the distribution of growth in the young sporophyll.

2. The Occurrence, Distribution, and Inception of Sori

The leaves of the young fern sporophyte are usually vegetative, the sori typically appearing on the leaves of mature plants. This fact, as Wardlaw

(1958) noted, suggests that there is a stage in the ontogeny that is marked by a critical physiological change and by a morphogenetic innovation which consists in the inception of the sporogenous meristems, or in a novel activation of existing growth centres. A cursory examination of the leaves of various mature ferns shows that not all of them bear sori, as is evidenced by *Todea*, *Osmunda*, *Dryopteris*, *Blechnum*, *Pteridium*, &c., and that any particular leaf is not necessarily fully fertile. In some species, the first leaf to unfold in spring or early summer may be entirely vegetative, as may also be the lower pinnae of the following leaves. Goebel (1930*a*) has recognized several soral (sporangial) distribution patterns, these being determined by factors in the genetical constitution and by various external factors. Thus the sori or sporangia may be (*a*) distributed over the entire leaf surface, or restricted to (*b*) the apical region, (*c*) the basal region, or (*d*) the central region of the frond only. Goebel attributed these differences to time relationships in the soral development. Wardlaw (1958) considered that both stimulatory substances and nutrients are likely to be involved in these developments; but work of any significance has yet to be done on this aspect. Goebel (1928) conjectured that the occurrence of sori and sporangia in particular positions on the sporophyll may be due to a localized accumulation of particular organic substances. This accumulation usually takes place at the end, or along the course of, a vein. When this hypothetical morphogenetic substance becomes more or less uniformly distributed between the veins, a corresponding spread of sporangium formation over the leaf surface—the so-called Acrostichoid condition—would tend to ensue (Goebel, 1930*b*, p. 1323).

An appreciation of the facts that the mature fern lamina is the outcome of the growth and meristematic activity of a distal and marginal meristems, and that these are organized regions, may lead to a better understanding of the phenomenon of soral inception. In a comparison of the marginal meristems of leaves with the shoot apical meristem, Wardlaw (1958) has noted that, like the latter, each marginal meristem comprises the distinctive (marginal) apical cell and the prism-shaped embryonic cells to which it has given rise. The further growth, division and differentiation of the prism-shaped cells yield the epidermal and mesophyll tissues, and there is a consequential thickening of the submarginal region of the leaf, very much like the marked widening of the subapical region of the shoot. Incipient vascular tissue, formed in proximity to the marginal apical cell, extends backwards, to join other similarly formed strands and eventually the mid-rib of the lamina. In short, to understand soral inception, it is necessary to understand something of the histological constitution of the whole marginal meristem.

3. Material and Methods

In the present investigation selected leptosporangiate ferns with discrete superficial sori were used including *Dryopteris dilatata* (*aristata*), *Phyllitis scolopendrium*, *Athyrium filix-femina*, *Cryptogramma crispa*, and *Polystichum aculeatum*, i.e. locally and easily available species. Plants with coiled leaves

were collected during two growing seasons, in 1956 and 1957. Fertile leaves, still at the coiled, 'crozier' stage, were collected from early March till the end of May at regular weekly or fortnightly intervals. Fertile portions of adult leaves were also collected between May and September to admit of observations on mature soral structures. Sporophylls of *Dryopteris filix-mas*, *Polypodium vulgare*, *Asplenium trichomanes*, *Asplenium rutamuraria*, and *Pteridium aquilinum* were collected for certain other investigations. Seemingly sterile regions of the leaf immediately subjacent to visible sori were also collected to see if dormant, inhibited, or vestigial sporogenous tissues could be distinguished. In *Dryopteris dilatata* (*D. aristata*) younger leaves, belonging to the group due to uncoil during the next growing season, were also collected at 10–12-day intervals from January to September (1957). The scaly investment was usually removed to allow effective penetration of the fixative. Fixing, embedding and staining were in conformity with common laboratory practice. Serial sections were cut in various planes, those in the vertical longitudinal plane of the crozier being most instructive.

4. Observations on the Inception and Eventual Position of Sori

(*a*) *Cryptogramma crispa* (L.) R.Br. (Parsley Fern)

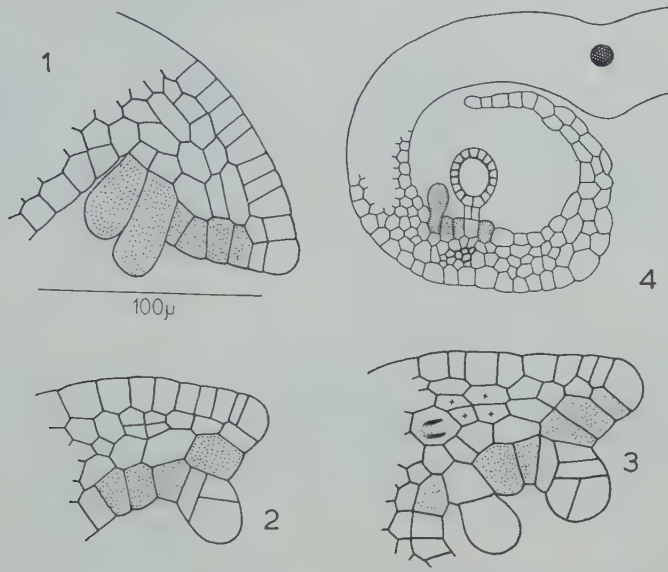
The rhizome of this relatively primitive Gymnogrammoid fern bears dimorphic fronds. The outer, sterile fronds are spreading and pinnate, the pinnae being further divided. The inner fertile fronds are upright, have longer petioles, and are twice pinnate. The ultimate segments have their margins strongly inrolled. The sori are superficial, are without indusia, and are elliptical in outline.

The marginal origin of the sporogenous meristem is shown in Text-figs. 1–3. Small groups of the distinctive prism-shaped cells of the abaxial marginal meristem begin to function at an early stage in the development of the sporophyll as sporogenous meristems. As the illustrations show, sporangium formation begins while the sporogenous meristem is still an integral part of the embryonic marginal tissue. However, during the further development of the sorus the marginal meristem remains active and adds a considerable amount of new tissue to the lamina. As a result, the maturing sorus eventually occupies a superficial position, remote from the margin, Text-fig. 4 and Plate 1, Fig. 1. The extended leaf margin becomes incurved, thus affording protection to the young sorus. Although the adult sorus is usually 'mixed', there is evidence of some regularity in the origin and early development of the sporangial initials in a young state, Text-figs. 1–3. The sporogenous meristem also contributes to the basipetal inception of the soral pre-vascular tissue.

(*b*) *Polystichum aculeatum* (L.) Roth.

The sori on the bipinnate leathery fronds are small and usually confined to the upper half of the leaf and are situated superficially at a considerable distance from the margins. In fact, they form a line on either side of the

mid-rib. Each sorus has a central peltate indusium. Although the earliest stages of soral development were not observed because of the scarcity of young sporophylls, sufficient evidence was obtained to show that the sporogenous meristem originates in the marginal meristem (Text-figs. 5, 6). The peltate indusium develops very early and overarches the margin of the young sporophyll and the sporogenous meristem. The cells of the latter divide longitu-



TEXT-FIGS. 1–4. *Cryptogramma crispa.*

FIG. 1. Section through the margin of a fertile segment, showing the origin of the prism-shaped cells of the sporogenous meristem (stippled) in the marginal meristem. The sporangial initials already show a conspicuous enlargement, i.e. before segmentation. The soral vascular strand is not yet discernible.

FIGS. 2, 3. Sections as in Fig. 1, showing the relationship of the prism-shaped cells of the sporogenous meristem to the marginal meristem. The inception of the soral vascular supply (marked x) can be discerned below the sporogenous meristem.

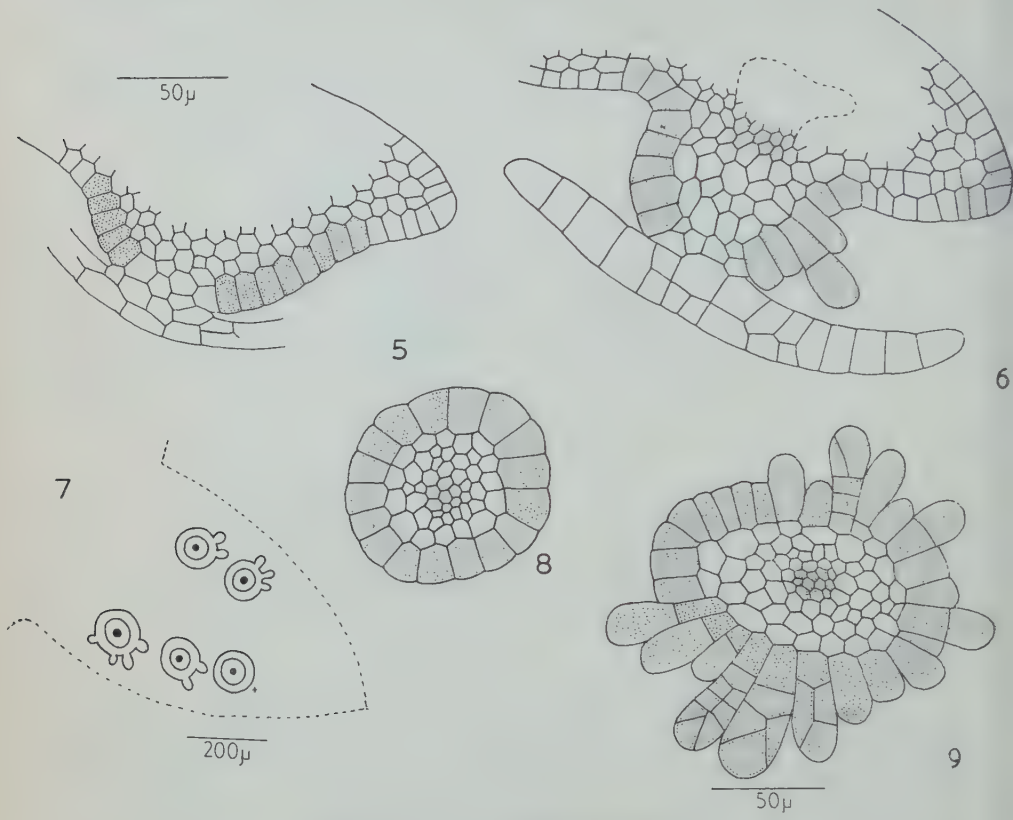
FIG. 4. Section of part of an adult fertile segment, showing the distinctly superficial sorus. The characteristic prism-shaped meristematic cells of the sporogenous meristem are readily distinguished, but the latter is now situated at a considerable distance from the margin where it originated. The soral vascular tissue is seen below the nascent sorus.

dinally and transversely with the result that it can accommodate additional sporangia upon it (Text-figs. 7, 8). The sporangia have their inception when the sorus is still near the leaf margin. As a result of sustained growth in the marginal meristem, the adult 'mixed' sorus eventually occupies a superficial position, Text-fig. 9.

(*c*) *Athyrium filix-femina* (L.) Roth.

In the tripinnate leaves, two short rows of sori are present on the lowest pinnulets, Text-fig. 10. The lowest sori of each row are kidney-shaped, horse-shoe-shaped, or more or less hooked, like those of *Dryopteris*; the upper ones are nearly or quite straight, oblong, or linear. The indusium is in conformity

with the shape of the sorus and is attached on one side. The kidney-shaped form of the basal sori was regarded by Bower (1928) as evidence of their 'conservatism'. In suitable vertical longitudinal sections of the young and



TEXT-FIGS. 5–9. *Polystichum aculeatum.*

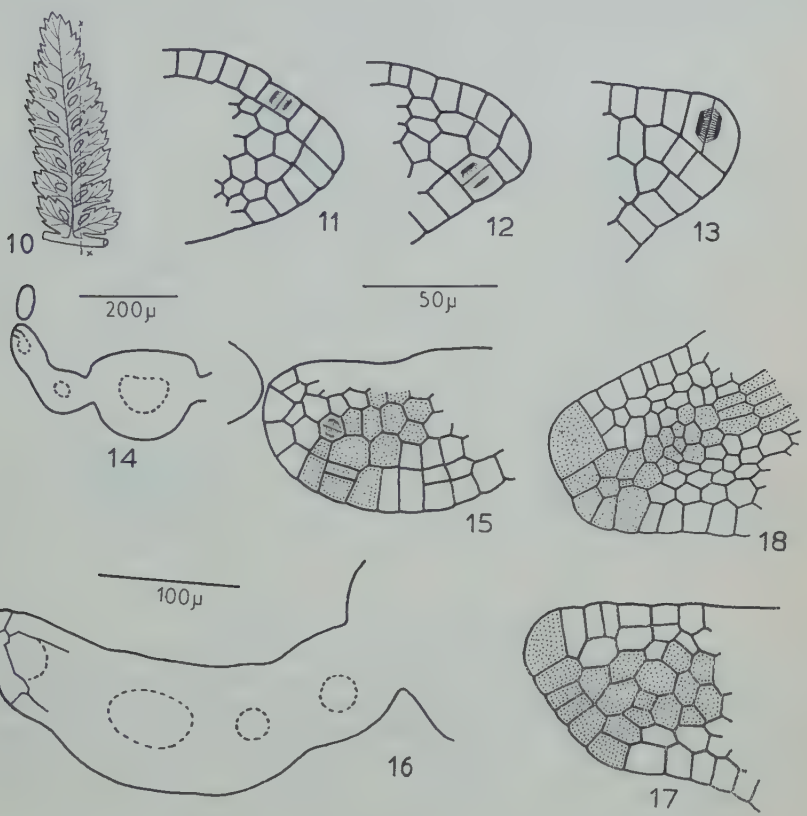
Fig. 5. Section through the margin of a fertile pinnule, showing the marginal apical cell and the prism-shaped cells of the sporogenous meristem (stippled) which constitute part of the marginal meristem. A peltate indusium (only part is shown) has grown out from a median position in the meristem.

FIG. 6 shows the well-developed sporogenous meristem still close to the leaf margin. Some further marginal growth has now taken place and the nascent sorus is superficial.

FIG. 7 is a diagrammatic representation of a section of a fertile pinnule, parallel to the abaxial surface. Two rows of sori can be seen. Figs. 8 and 9 show details of the nascent sori seen in such sections. The distinctive prism-shaped cells of the sporogenous meristem encircle a central mass of parenchymatous tissue, the innermost cells of which are becoming differentiated as the soral vascular strand. In Fig. 9 some cells of the sporogenous meristem have recently divided by longitudinal walls, thereby increasing the surface available for sporangium formation. Sporangia at various stages of development can also be seen.

coiled leaves, it can be seen that the marginal apical cell cuts off segments alternately right and left (Text-figs. 11–13). These divide by periclinal and anticlinal walls, and remain in the embryonic state, so constituting the marginal meristem. When the reproductive phase becomes incident, sporogenous

meristems originate in the abaxial marginal meristem, Text-figs. 14–18. The cells of the sporogenous meristem are seen to be in communication with an older vascular strand of the sporophyll through some basally derived, meristematic cells. The prism-shaped cells of the sporogenous meristem divide both periclinally and anticlinally, thereby adding to the surface area and thick-



Text-figs. 10–18. *Athyrium filix-femina.*

Fig. 10. Typical pinnule, showing venation and sori.

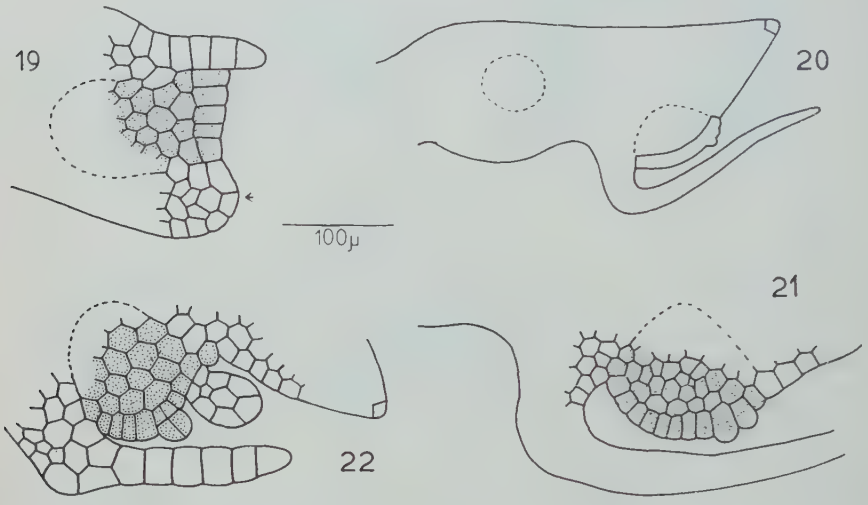
Figs. 11–13. Typical transverse sections through the margins of fertile pinnules, showing divisions in the apical cell and adjacent prism-shaped cells of the marginal meristem.

Figs. 14, 15. Transverse section of a fertile segment, showing the position of inception of the sporogenous meristem (stippled) in the marginal meristem. A nascent soral vascular strand is becoming confluent with an older vascular strand of the segment.

Figs. 16, 17, 18. Further evidence of the position of origin of the sporogenous meristem and the inception of its basipetally derived vascular strand. (Figs. 11–13, 15, 17, 18 all to same scale).

ness of the tissue, Text-fig. 19; and further cell divisions raise the meristem slightly above the surface of the segment, Text-figs. 20, 21. At the same time, contact with the older vascular strand below is maintained through the soral pre-vascular supply. Text-figs. 19–22 illustrate the inception of the indusium. The indusial initial is the cell of the sporogenous meristem farthest from the

margin of the sporophyll. Subsequent divisions lead to the formation of a flap-like indusium which is usually one cell thick, except at the base which is two-celled. As a result of the continued growth of the marginal meristem, the sporogenous meristem becomes progressively displaced so that the sorus finally occupies a distinctly superficial position (Text-fig. 22). Though there are indications of an initial 'gradate' sequence of sporangial development, the sorus eventually becomes 'mixed', as in *Dryopteris dilatata.*



TEXT-FIGS. 19–22. *Athyrium filix-femina.*

FIG. 19. Transverse section of a fertile segment showing the development of the sporogenous meristem and the beginning of indusium formation (arrow). The distinctive prism-shaped cells of the sporogenous meristems can be seen in close proximity to the leaf margin.

FIGS. 20, 21. Diagram and more highly magnified portion of a section of a fertile segment, showing the relative displacement of the sporogenous meristem on to the abaxial surface as a result of the continued growth of the leaf margin. The indusium is now well developed, and sporangium formation is beginning. Fig. 22 illustrates a slightly later stage in the development of the superficial sorus.

(*d*) *Dryopteris dilatata* (Hoffm.) A Gray (*D. aristata* (Vill.); *D. austriaca*)

The superficial sori are numerous, round, and disposed in two rows on the pinnulets, one on either side of the mid-rib. Each sorus is situated on the acroscopic fork of a secondary vein. The indusium is reniform to rounded, prominent and convex. The sorus in *Dryopteris* is sometimes misunderstood, chiefly because sections tend to be cut transversely to the vein. In such sections the indusium appears to have a central stalk and to overarch the receptacle equally on all sides. A more accurate conception is gained from sections which follow the course of the vein, and cut the sorus in the median plane of its dorsiventrality: the sporogenous meristem is now seen to be asymmetrical from the outset, orientated towards the leaf margin, with the indusium protruding from its top.

When the sporophyll is cut parallel to its abaxial surface, the sorus is seen to be composed of distinctive prism-shaped embryonic cells encircling polygonal, parenchymatous cells, the innermost cells of which in due course differentiate into the elements of the soral strand (Plate 1, Fig. 2). The mature sorus is discrete and superficial, and situated some distance from the margin. At its inception, the sporogenous meristem can be seen to originate from the marginal meristem (Plate 1, Figs. 5–7). The prism-shaped cells of the sporogenous meristem are here slightly raised as a result of their periclinal divisions leading to the early formation of the ground tissue of the receptacle. Plate 1, Figs. 3, 4 illustrate the inception of sporogenous meristems in the horizontal plane of the sporophyll and their intimate connexion with the associated pre-vascular tissue. The fertile meristem, while still close to the leaf margin, now grows out as a prominent mound-like form. The mid-rib of the segment is still at an early stage of differentiation. The indusial outgrowth slightly protrudes beyond the sporogenous tissue (Plate 1, Fig. 7) but the soral strand is not yet differentiated. The basipetal soral pre-vascular strand now becomes conjoined with older vascular strands of the fertile segment (Plate 1, Fig. 3). The pre-vascular tissue has elongated cells, devoid of starch and chlorophyll and with prominent nuclei. On the further marginal growth of the fertile segment, the sporogenous meristem occupies positions progressively more remote from the margin, so that the adult sorus is eventually superficial. The sporangia are formed in a 'mixed' sequence with the kidney-shaped indusium originating from the top of the sporogenous meristem.

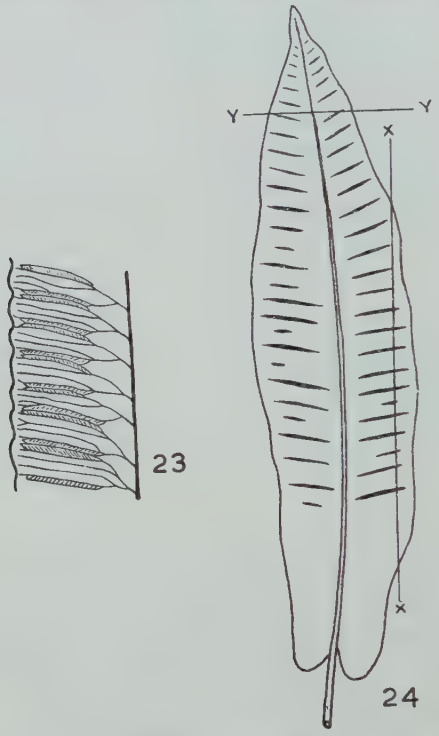
(*e*) *Phyllitis scolopendrium*

The sori are linear and usually more abundant in the distal part of the frond; in vigorous plants they occupy almost the whole width of the blade from mid-rib to margin, though usually less; the sporangia of the paired sori typically run together to form a dense brown cigar-shaped mass. The relation of the sori to the sinuous leaf margin is shown in Text-fig. 23. The sori associated with the successive primary veins face one another—the paired sori characteristic of this genus. A slight indentation of the sinuous margin corresponds with each pair and a convexity with the region between the forks of the primary veins.

The increase in size of the lamina results from the meristematic activities of the distal and marginal meristems. Each marginal meristem comprises a distinctive (marginal) apical cell and the prism-shaped embryonic cells formed from it. The further growth, division and differentiation of the latter give rise to the epidermal and mesophyll tissues, and there is a consequential thickening of the submarginal region of the lamina.

Serial sections of very young sori, i.e. in small circinately coiled leaves, were cut parallel to and at right angles to the mid-rib, Text-fig. 24. In the former and proceeding from the margin inwards, i.e. cutting the linear sori transversely, evidence of all stages of soral development, from its inception to the adult structure, was obtained. Vertical longitudinal sections of the sorus, i.e.

at approximately right angles to the mid-rib, show how the sporogenous meristem is related in its origin to the marginal meristem (Plate 2, Figs. 8, 13). The sporogenous meristem, originating from the marginal meristem, is composed of distinctive prism-shaped cells which are longer than the adjacent superficial cells (Plate 2, Fig. 13). The slight depression (Plate 2, Fig. 9), characteristic of the soral locus, is visible with the initial cell of the sporogenous meristem containing a medianly-placed nucleus, the adjoining region



TEXT-FIGS. 23, 24. *Phyllitis scolopendrium* (diagrammatic).

FIG. 23. Portion of a lamina, with mid-rib, showing the relationships between the venation, the position of the sori and the marginal sinuosities. (Redrawn from Bower.)

FIG. 24. Diagram showing the two planes in which the sections were cut; X—X, parallel to the mid-rib and at right angles to the sorus; Y—Y, at right angles to the mid-rib traversing some sori lengthwise.

being two cells deep. This initial cell is evidently one of the marginal meristem cells. It divides anticlinally and gives rise to two daughter cells. Successive anticlinal and periclinal divisions result in the organization of a distinctive, sunken, sporogenous meristem (Plate 2, Fig. 10). The intersoral region, on the other hand, shows no such innovation. Further anticlinal and periclinal divisions of the embryonic cells lead to an increase in the volume and surface area of the sporogenous meristem (Plate 2, Fig. 11). The soral depression has by now become quite conspicuous as a result of increased segmentation in

different planes and enlargement of the adjacent cells; these give rise to the indusial initials (Plate 2, Figs. 11, 12). Evidence of the soral pre-vascular tissue can be seen on one side of the young sorus. The indusial initial undergoes segmentation (Plate 2, Fig. 12) fairly early in the formation of the sorus in the usual manner to give rise to a typical flap-like structure. Sporangial development does not take place until the sporogenous meristem has come to occupy a superficial position at some distance from the leaf margin. Further development of the paired sori follows the same lines as in *Blechnum punctulatum* var. *krebsii* (Bower, 1914), with minor differences of detail. As Burck (1874) has already dealt with them, only a very brief account need be given. The incipient soral vascular strands develop right and left below the indusial flaps of the paired sori. The region between them is devoid of vascular tissue and the central region of the sporogenous meristem rises into a sterile ridge. The indusial flaps have meanwhile enlarged, being typically a single layer in thickness, except at their bases. They overlap very fully and protect the sporogenous meristems which are eventually situated on either side of the base of the ridge and immediately above their associated vascular strands. The latter are usually larger than the intersoral strands. Though indications of a basipetal succession of sporangial development may be seen in early stages, the sorus later becomes distinctly 'mixed' in character.

(*f*) *Inhibited or Non-functional Sporogenous Meristems in Vegetative Pinnules*

With the concept of the soral site as a sporogenous meristem in mind, it would be interesting, as Wardlaw (1958, 1961) has pointed out, to know if such meristems are also present but inhibited, non-functional, or lacking some necessary developmental stimulus, in purely vegetative leaves, or in non-fertile pinnae or pinnules in otherwise fertile leaves.

Vegetative segments and sterile areas of sporophylls of *Polypodium vulgare*, *Dryopteris filix-mas*, *Dryopteris dilatata*, *Asplenium trichomanes*, *Pteridium aquilinum*, and *Phyllitis scolopendrium* were examined closely after preparing transverse sections. No evidence of dormant or vestigial sporogenous meristems was found in these vegetative areas. The abaxial epidermal cells showed an advanced parenchymatous differentiation and contained large starch grains. By contrast, the fertile tissue in a sporophyll usually has the appearance and characteristic staining of meristematic or semi-meristematic tissue and there are no starch grains present. Abortive soral sites, however, did resemble rudimentary meristematic regions, but in such cases the soral loci were evident to the naked eye.

5. Discussion

The point has been made that there is unity of meristematic tissue throughout the fern plant, the apical and marginal meristems of leaves being referable to the shoot apical meristem in which the leaf primordium originates (Wardlaw, 1958). Fern sori, which may be situated on or within the leaf margin, or on the lower surface of the sporophyll, originate from localized areas of embryonic tissue. Each soral site, which may be regarded as a region with special

metabolic properties, is capable of giving rise to specific organs of reproduction. In the present investigation, the idea has been examined that the embryonic soral locus, receptacle, or placenta, is a special kind of meristem, in fact, a *sporogenous meristem*. The evidence presented shows that this idea is demonstrably valid. Thus, whereas Bower regarded the superficial sorus as being due to a 'phyletic slide', i.e. to a sequence of evolutionary changes, from an original marginal position characteristic of more primitive ferns, we can now see that it is an ontogenetic process in living species: it is, in fact, referable to a growth centre initially located in the leaf marginal meristem. In some ferns, the inception of soral sites in the leaf marginal meristem results in a correlative inhibition of the growth of the latter and the leaf matures with marginal sori. But in other ferns the marginal meristem retains its capacity for growth and, as development proceeds, the nascent sorus comes to occupy a more or less conspicuously superficial position. Since these different growth relationships between sporogenous meristems and the marginal meristems of different species are ultimately due to genetical factors, we begin to see how the results of morphogenetic inquiry may eventually have an important impact on, and convergence with, phylogenetic views.

In the young fern sporophyte the leaves are usually vegetative, the sori typically appearing on the leaves of older plants which have reached the adult state. This fact, as Wardlaw (1958, 1961) has noted, suggests that there is a stage in the ontogeny which is marked by critical physiological changes in the leaves, and by attendant morphogenetic developments, in particular, the inception of the sporogenous meristems. This development in ferns is reminiscent of the onset of flowering in dicotyledons and monocotyledons and may be due to closely comparable causes, though this aspect has so far received little attention (for a review of the literature, *see* Wardlaw, 1961). During the vegetative phase, no evidence has been obtained of the presence in the leaf margin of dormant or inhibited sporogenous meristems, or soral sites: the abaxial marginal meristem cells, after a phase of division, simply enlarge into the parenchymatous tissues of epidermis and mesophyll. The effects of age, environmental factors, and of various experimental treatments, on the onset of reproduction will be considered in a later paper.

Since each sorus, e.g. in *Dryopteris dilatata*, *Athyrium filix-femina*, &c., originates from a marginal meristem, it is also formed in close proximity to the associated vein. On the basis of the observations described and illustrated in the present work and in earlier investigations, it can be seen that the sporogenous meristem gives rise to a short vascular strand—the receptacular vascular supply—which soon becomes conjoined with the vein associated with the parent marginal meristem. Moreover, in species where the sporogenous meristem becomes extended along the leaf margin, or over the lower surface of the sporophyll, there is a corresponding distribution of soral vascular tissue. Goebel (1930*b*) discussed the nature of the stimulus that leads to these distinctive vascular developments in sporophylls, and concluded that the primary stimulus comes from the sorus. The morphogenetic situation, as Wardlaw

(1958) has noted, is probably considerably more complex than has been thought: a sorus may be envisaged as originating as a growth centre in a leaf marginal meristem, presumably in relation to an outward, or acropetal, movement along the associated vein of some special substance—the 'sporogenous substance'—formed in the older leaves or older regions of the plant. During its normal vegetative growth and meristematic activity, the leaf marginal meristem gives rise to a vein, along which metabolic substances may move in both directions; with the inception and active development of a sporogenous meristem, a strand of soral vascular tissue becomes differentiated.

Wardlaw (1958) has pointed out that in respect of its position of origin, regulated development, and organization, the sporogenous meristem of a superficial sorus, such as those studied here, bears the same general relation to its parental leaf marginal meristem as a leaf primordium does to the shoot apical meristem. The individual, discrete, sorus of *Dryopteris dilatata*, for example, originates in the leaf marginal meristem at some distance from the marginal initial cell; it develops as a structure of dorsiventral symmetry, with its indusium orientated towards the leaf margin from which it originates; and it is vascularized. Such observations are of interest in that they suggest that certain functional activities, or organizational features, may be common to all superficial meristems, roots excepted.

In conclusion, a survey of the evidence indicates that all sori are of marginal origin, various relationships between the growth of the leaf marginal meristem and the sporogenous meristem determining the eventual position of the sorus on the mature leaf. Environmental factors, the stage reached in the ontogeny, leaf growth including various correlative developments, and genetical factors, are all involved in the inception, development, and positional relationships of sori and sporangia. Although the 'spore-producing members' in the ferns have been the subject of classical investigations on the part of distinguished comparative morphologists, it is maintained that fuller morphogenetic investigations of these organs are likely to be rewarding from both the phyletic and the causal points of view.

The authors are grateful to Messrs. E. Ashby and G. Barker for the photographic illustrations.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. *Cryptogramma crispa.* Cross-section of a young fertile segment, showing the 'displacement' of the sporogenous meristem as a result of sustained growth in the marginal meristem. (×250)

Fig. 2. *Dryopteris dilatata.* A young sorus as seen in a section cut parallel to the abaxial surface of a pinnulet, showing the distinctive prism-shaped cells of the sporogenous meristem surrounding the central tissue. The soral strand has not yet become differentiated. (×500)

Fig. 3. *Dryopteris dilatata.* Part of a horizontal section of a fertile segment, illustrating the histology of the sporogenous meristem at its inception. An incipient vascular strand connects this meristem with the vein of the pinnulet. (×400)

Fig. 4. *Dryopteris dilatata.* Part of a horizontal section of a pinnulet, showing the prism-shaped cells of the sporogenous meristem and associated pre-vascular tissue which basipetally becomes conjoined with the older vascular tissue of a vein. The pre-vascular tissue consists of elongated cells without starch or chloroplasts. (×400)

Fig. 5. *Dryopteris dilatata.* Vertical transverse section of the marginal region of a young fertile pinnulet, showing the position of inception of the prism-shaped cells of the sporogenous meristem (arrow); i.e. in the marginal meristem. At this stage the sporogenous meristem comprises only a few cells—it is a growth centre—but already these are beginning to be slightly raised above the level of the adjacent tissue. (×400)

Fig. 6. *Dryopteris dilatata.* Like Fig. 5, but at a slightly later stage, showing the prominent mound-like sporogenous meristem (arrow) still constituting part of the marginal meristem. (×400)

Fig. 7. *Dryopteris dilatata.* As in Figs. 5 and 6, but at a more advanced stage, showing the sporogenous meristem (arrow) still close to the margin and (below) the indusial outgrowth projecting somewhat beyond the fertile tissue. The soral strand is not yet differentiated and the xylem elements of the mid-rib are still not lignified. (×400)

Plate 2

Fig. 8. *Phyllitis scolopendrium.* Section through a leaf margin at right angles to the mid-rib (indicated in Text-fig. 24 as section Y—Y), showing the active segmentation in the marginal meristem and a consequential thickening of the sub-marginal region. (×500)

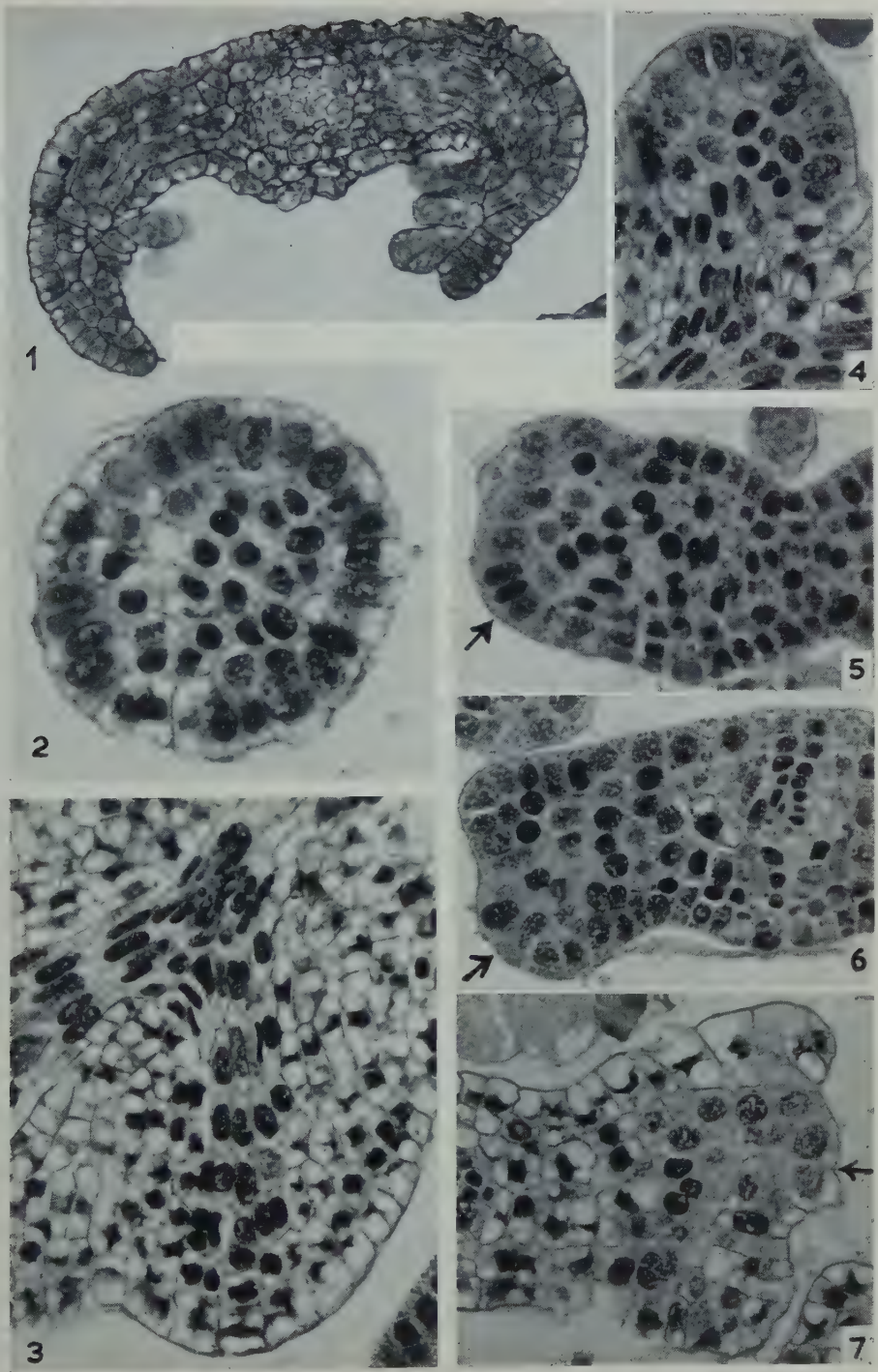
Fig. 9. *Phyllitis scolopendrium.* Section of a sporophyll parallel to the mid-rib (indicated in Text-fig. 24 as section X—X) showing the inception of a sporogenous meristem (arrow). The single 'fertile' marginal cell differs in size and appearance from the adjacent cells. (×500)

Fig. 10. *Phyllitis scolopendrium.* Section of a sporophyll as in Fig. 9, showing the conspicuous soral depression and the development of the sporogenous meristem; one of the constituent cells is dividing anticlinally. (×500)

Fig. 11. *Phyllitis scolopendrium.* As in Figs. 9, 10: a later stage in the organization of the sporogenous meristem showing the indusium initials. One of the sporogenous meristem cells is dividing periclinally. A soral vascular strand is becoming differentiated. (×500)

Fig. 12. *Phyllitis scolopendrium.* As in Fig. 11: a still later stage in the organization of the sporogenous meristem. One of the constituent cells is dividing anticlinally, and the indusium is beginning to be formed. (×500)

Fig. 13. *Phyllitis scolopendrium.* Section of the margin of a sporophyll (cut in the plane Y—Y in Text-fig. 24) showing that the sporogenous meristem (arrows) originates from the marginal meristem. Here the sporogenous meristem is cut along its length. It is composed of distinctive prism-shaped cells with densely-staining nuclei. (×500)



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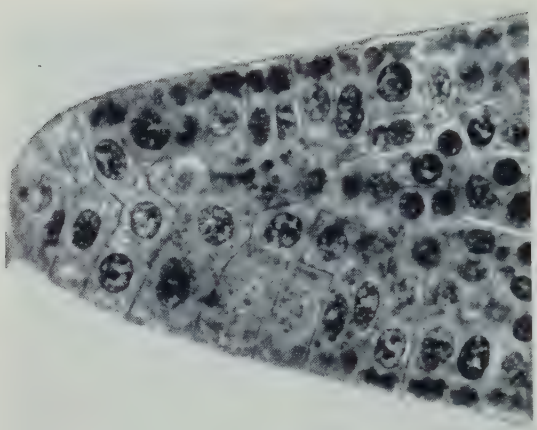

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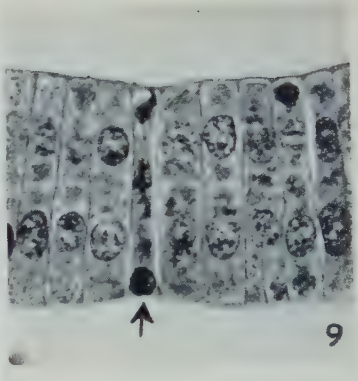

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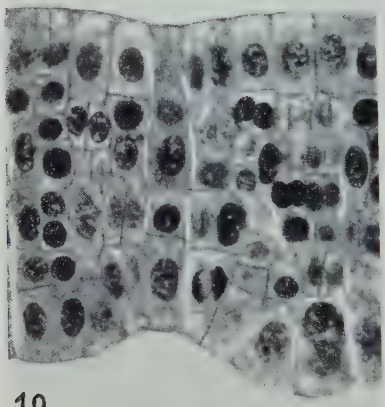

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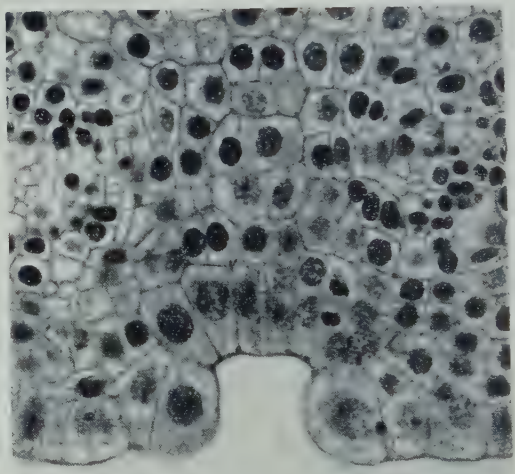

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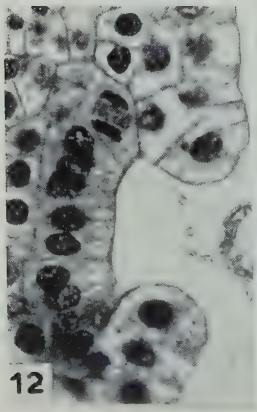

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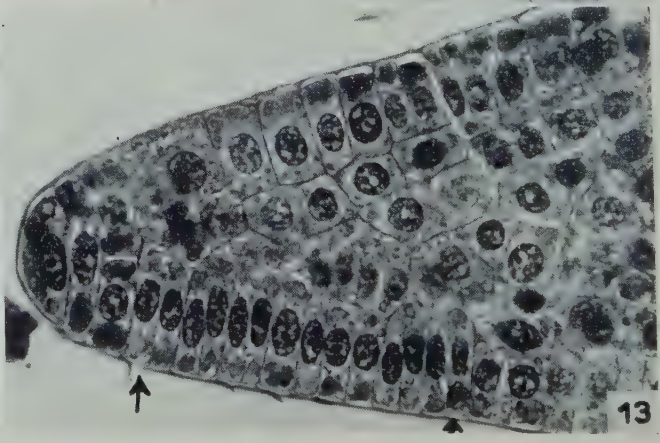

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γ-Aminobutyric Acid Metabolism in Plants

Part 1. Metabolism in Yeasts

BY

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With five Figures in the Text

Abstract

The metabolism of γ-aminobutyric acid (γAB) by two yeasts, *Saccharomyces cerevisiae* and *Torulopsis utilis*, was investigated. Both yeasts grew well upon γAB as a sole source of nitrogen (N), and the lag phase for *Torulopsis* was shorter than when NH_4^+ provided the N-source. The metabolism of γAB by *Torulopsis*, which was associated with an increased O_2 uptake, was adaptive in character. The enzyme whose formation was induced by the supply of γAB was a transaminase, which was apparently specific for γAB as the amino donor. Small amounts of transaminase were present in unadapted, NH_4^+-grown cells. The optimum pH, equilibrium constant, Michaelis' constant, and coenzyme requirement were investigated for the transamination reaction involving α-ketoglutaric acid (αKG) as amino group acceptor. Succinic semi-aldehyde (SSA) was a product of this transamination reaction. The possibility that some γAB was converted into SSA by a direct oxidative deamination remained unconfirmed.

The further conversion of SSA into succinic acid was established using intact cells for both yeasts. This oxidation process was shown to be linked to the reduction of pyridine nucleotides using extracts of *Saccharomyces* as a source of SSA dehydrogenase. Dehydrogenase activity could be ascribed to two separate enzymes, one linked to DPN, and the other utilizing TPN and requiring Mg^{++} as an activator. The properties of the former enzyme, which was more important quantitatively, were investigated and compared with those described in the literature for an aldehyde dehydrogenase of baker's yeast and for SSA dehydrogenases of *Pseudomonas*. *Torulopsis* extracts could catalyse the reduction of SSA to γ-hydroxybutyric acid (γOHB); the γOHB dehydrogenase involved required TPNH as a coenzyme. Certain other properties of this enzyme are recorded.

The possibility is discussed that γAB and SSA act as intermediates in a metabolic pathway that may form a by-pass of the αKG-succinate stage of the tricarboxylic acid cycle.

Introduction

THE investigations to be described in this and the succeeding paper were commenced some years ago when there were few studies on the metabolism of γ-aminobutyric acid (γAB) in plants. Part 1 deals with the metabolism of γAB in two yeasts, *Torulopsis utilis* (food yeast) and *Saccharomyces cerevisiae* (baker's yeast). The results are of some value in comparative plant biochemistry although they can now no longer claim originality following the recent publications of Jakoby and his co-workers upon the enzyme systems involved in γAB and succinic semi-aldehyde (SSA) metabolism (Scott and Jakoby, 1959; Jakoby and Scott, 1959; Nirenberg and Jakoby, 1960). Higher

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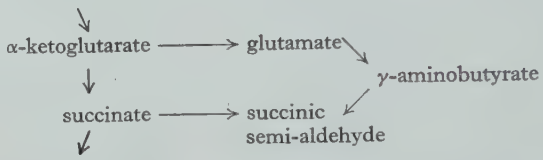
plant tissues were used in the studies described in Part 2 which provide the first decisive proof that γAB can undergo transamination in angiosperms, and that this may be the primary reaction that releases the carbon-skeleton for further degradative metabolism.

By the use of paper-chromatographic techniques, γAB has been recognized as a constituent of nearly every plant examined and it is present frequently as a major component of the free amino-acid pool. Reed (1950) demonstrated that γAB was a normal constituent of *Saccharomyces* and isolated the amino-acid from commercial yeast extract.

γAB probably arises largely by decarboxylation of glutamic acid, although it has been established clearly that no correlation exists between the amounts of γAB and the activity of glutamic decarboxylase present in plant tissues (Thompson, Pollard, and Steward, 1953; Fowden, 1958). γAB, together with SSA, have been implicated as intermediates in a postulated reaction scheme that could serve as a by-pass mechanism of the α-ketoglutaric acid (αKG)-succinic acid stage of the tricarboxylic acid-cycle reactions (Bessman, Rossen, and Laynes, 1953; Naylor and Tolbert, 1956; Fowden, 1958):



γAB would yield SSA either by transamination or by direct oxidative deamination, and upon further oxidation the aldehyde could give succinate.

The data to be presented in Part 1 provide nutritional and enzymic evidence supporting the possible operation of this sequence of reactions in the two yeasts.

Materials and Culture Media

Food yeast. Pure cultures of *Torulopsis utilis* (I.M.I. 23311 from the Commonwealth Mycological Institute) were maintained on 2 per cent. malt extract–1·5 per cent. agar.

Baker's Yeast. Commercial pressed yeast (Distillers Company Ltd.) was washed thoroughly by centrifugation using nitrogen-free (N-free) medium.

Culture media. The normal complete culture medium (NH_4^+-medium) used to grow the yeasts contained: Sucrose 50 g., KH_2PO_4 1·5 g., K_2SO_4 1·5 g., $MgSO_4.7H_2O$ 1·0 g., $CaCl_2$ 0·05 g., $(NH_4)_2SO_4$ 2·0 g., $(NH_4)_2$ HPO_4 1·5 g., 1 ml. of a trace-element solution (Horowitz and Beadle, 1943), and distilled water to 1 l. The pH was adjusted to 6·2. Cells grown in this medium are termed NH_4^+-grown yeast.

γAB-grown yeast was produced using a modified medium (γAB-medium) in which γAB (5·4 g./l.) supplied all the medium-N; $(NH_4)_2$ SO_4 and $(NH_4)_2HPO_4$ were omitted from this medium and KH_2PO_4 was increased to 3·0 g./l.

An adaptation of the complete medium, where N was supplied as $(NH_4)_2 HPO_4$ 0·75 g./l., and γAB 2·7 g./l., gave a mixed NH_4^+-γAB-medium in which half the N was supplied as NH_4^+ salts and half as γAB.

All batches of yeasts were grown in media continuously shaken at 25°.

N-starved yeast was obtained by shaking cells in a medium (N-free medium) from which all N-containing substances were omitted.

Other experimental methods used will be outlined briefly as the related results are presented.

1. γAB METABOLISM

A. *The γAB Content of Yeasts*

γAB was shown to be a constituent of both yeasts by paper-chromatographic methods.

The tentative conclusion reached by Reed (1950) that the quantities of γAB present in baker's yeast increased during periods of anaerobiosis could not be confirmed. Approximately 2 g. quantities of freshly washed baker's yeast were transferred to two 150 ml. portions of NH_4^+-medium in 1 l. flasks and the yeast was grown for 12 hours with either air or nitrogen as the gas phase. The anaerobically produced yeast cells contained considerably more alanine but markedly less glutamic acid and γAB than did the aerobic yeast. The present findings are explicable if it is assumed that alanine and glutamic acid concentrations are governed by the rates of production of their keto-acid precursors, since anaerobiosis, by limiting the operation of the tricarboxylic acid cycle reactions, would lead to decreased concentrations of the acid intermediates, including αKG, whilst pyruvic acid would accumulate. The fall in γAB concentration during anaerobiosis indirectly supports the idea that it arises from glutamic acid.

γAB served as a good N-source for the growth of *Torulopsis* (see Section 1B). The free amino-acid composition of cells grown on the γAB-medium did not differ appreciably from that of other cells grown on NH_4^+-medium. When growth was upon γAB-medium, no abnormal intracellular accumulation of γAB occurred; indeed, the concentration of the acid in γAB-grown cells was lower than the corresponding level in NH_4^+-grown cells. These facts indicated that the amino group of γAB must freely enter the general system of intermediary nitrogen metabolism of *Torulopsis*.

B. *The Growth of Torulopsis on γAB*

Although γAB could function as a good sole source of N for the growth of *T. utilis*, no growth occurred in a medium in which γAB was the only carbon source.

The lag phase. Cells used in these experiments were grown initially on NH_4^+-medium. They were centrifuged aseptically and then shaken in N-free medium for 16 hours at 25° before being transferred at zero time into either NH_4^+-, γAB- or mixed NH_4^+-γAB-media. The growth-rate of the *Torulopsis* and the yield of cells (14–16 mg./ml. dry weight) after the cessation of growth

were the same in all media, but the length of the lag phase varied with the N-source used (see Fig. 1). The lag phase was shortest for γAB-medium and longest for NH_4^+-medium.

During the lag phase the concentrations of many nitrogenous metabolites (depressed during N-starvation) presumably must be restored to levels able to support renewed growth. Since determinations made in the first few

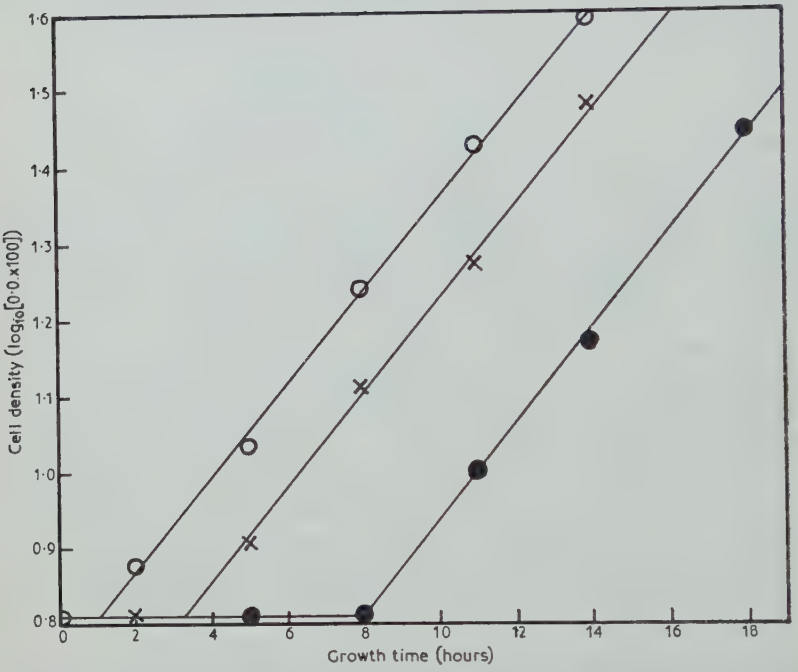


FIG. 1. Illustrates the rates of growth at 25° of *Torulopsis utilis* upon media, pH 6·2, containing various N sources as follows: o—o, γAB; x—x, mixture of γAB and NH_4^+; ●—●, NH_4^+.

minutes following the introduction of N-starved cells into various N-containing media showed that N entered the cells more rapidly from γAB-medium than from NH_4^+-medium, producing higher amide-N levels in cells supplied with γAB, the shorter lag phase observed for γAB nutrition received some experimental support.

When inorganic and organic N-sources were supplied simultaneously in the mixed NH_4^+-γAB-medium, NH_4^+-N entered the *Torulopsis* cells more rapidly than did γAB-N. This last finding is in agreement with observations summarized by Ingram (1955) which indicate that yeasts utilize NH_4^+-N in preference to N present in many amino-acids (tests upon γAB were not recorded) when both are supplied simultaneously.

C. *The Effect of γAB upon the Respiration of Yeast*

In these experiments washed cells of *Saccharomyces* or *Torulopsis*, previously grown in NH_4^+-medium, were shaken for 1 hour in 0·03 M. phosphate

buffer, pH 6·5, to reduce the level of endogenous respiration. After centrifugation, they were resuspended in phosphate buffer and aliquots were introduced in Warburg flasks. The rates of O_2 uptake by the cells were determined in the presence of γAB, αKG, and a mixture of the two acids using standard manometric techniques. The results obtained in one experiment using *Torulopsis* are illustrated in Fig. 2. Dry weight determinations made at the beginning and end of the experiment established that no growth occurred.

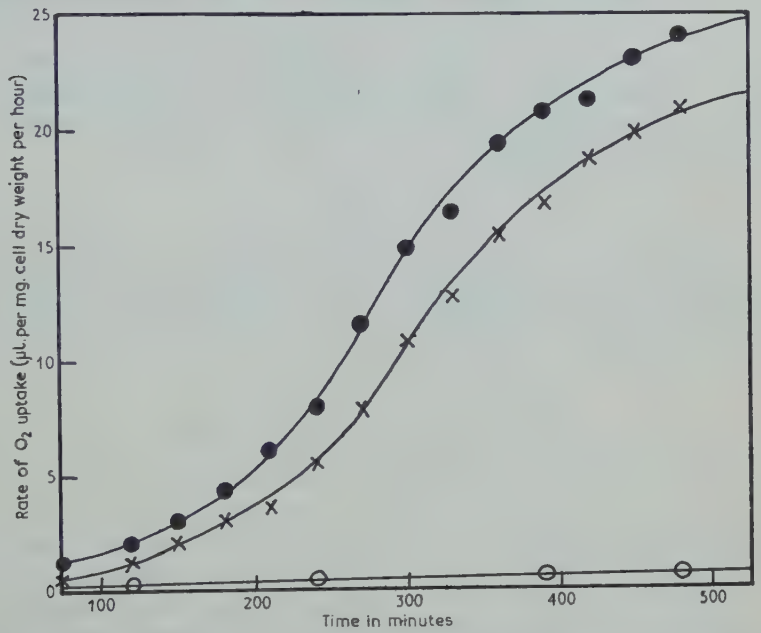


FIG. 2. Illustrates the rates of O_2 uptake at 25° of intact cells of *Torulopsis utilis* at pH 6·5 in the presence of the following substances: o—o, 0·02 M. αKG; x—x, 0·02 M. γAB; ●—●, 0·02 M. αKG+0·02 M. γAB.

During an initial period (from about 1 to 2·5 hours in different experiments) after the supply of γAB to cells, their rate of O_2 uptake remained low and constant. Subsequently, the respiratory rate in the presence of γAB gradually increased until it reached a maximum value 8 to 12 hours later. The value of Q_{O_2} (µl. O_2 uptake/mg. dry weight cells/hour) obtained finally was constant provided γAB and O_2 were not limiting. When the supply of γAB was exhausted, the respiratory rate fell. These results suggest that one or more of the reactions involved in the metabolism of γAB are oxidative and that at least one enzyme involved in the early stages of γAB utilization is adaptive in character, although this enzyme need not be concerned with an oxidative reaction.

The enzyme whose formation was induced by the presence of γAB was fairly labile, and, after the exhaustion of γAB, the Q_{O_2} of adapted cells kept overnight at 25° had returned to a level comparable with that observed for

the cells during the first hour of the previous day's experiment. The sequence of adaptive changes observed on the first day was reproduced when a further quantity of γAB was given to the cells; however, the lag phase was either much shorter or completely eliminated.

The rate of O_2 uptake by *Torulopsis* cells in the presence of αKG did not increase with time and was only slightly higher than that of endogenous respiration. However, when αKG was supplied together with γAB, O_2 uptake was appreciably higher than the combined total obtained when αKG and γAB were used singly. Presumably an interaction (probably transamination, see Section 1D) occurred between the two substrates yielding compounds that could be respired further. A similar situation was encountered when γAB and αKG were respired by brain tissue (Sugiura, 1957) and by *Achromobacter* (Higashi, Horio, and Okunuki, 1957).

D. *The Deamination of γAB*

It has been demonstrated already that γAB was deaminated readily when supplied to yeasts. An amino-acid oxidase (reaction (1)) or a transaminase (reaction (2)) probably catalysed the reaction. SSA would be a product of

$$\gamma\text{AB}+\tfrac{1}{2}\text{O}_2 \rightarrow \text{SSA}+\text{NH}_3. \qquad (1)$$

$$\gamma\text{AB}+\alpha\text{KG} \rightarrow \text{glutamic acid}+\text{SSA} \qquad (2)$$

$$\text{glutamic acid}+\tfrac{1}{2}\text{O}_2 \rightarrow \alpha\text{KG}+\text{NH}_3 \qquad (3)$$

If αKG was used as the acceptor keto acid in the transamination reaction (2), the glutamic acid produced could be oxidized via the glutamic dehydrogenase-diphosphopyridine nucleotide (DPN)-cytochrome system according to the overall reaction (3). Since the net result of the operation of reaction (2) followed by reaction (3) is the same as reaction (1), the deamination of γAB probably will be associated with extra O_2 uptake irrespective of whether a transaminase or an amino-acid oxidase catalyses the primary reaction. The succinic semi-aldehyde produced may undergo further oxidative degradation (see Section 2A).

Direct oxidative deamination. No definite conclusion has been reached concerning the presence of a γAB oxidase in yeast. If a system continues to metabolize an amino-acid with associated O_2 uptake, even in the presence of arsenite (which inhibits the oxidation of keto acids), then a direct oxidative deamination of the amino-acid is often assumed to have occurred. In our experiments, whole cells and non-dialysed, cell-free extracts of *Saccharomyces*, previously adapted to γAB utilization, continued to metabolize γAB with associated O_2 uptake even in the presence of arsenite. The O_2 uptake associated with γAB metabolism by whole cells was reduced to about half its initial value when 0·005 M. arsenite was present. Analogous experiments performed with adapted *Torulopsis* cells gave variable results when treated with arsenite.

The fact that a proportion of the respiratory O_2 uptake is insensitive to arsenite does not provide a crucial demonstration of a γAB oxidase, because it seems highly probable that αKG and glutamic dehydrogenase were present in all systems tested, and so residual O_2 uptake in the presence of arsenite may have been due to reactions (2) and (3), which do not involve oxidation of a keto acid, and not to reaction (1). The lability of the system responsible for O_2 uptake in the presence of γAB makes it difficult to devise an experimental procedure that would distinguish between the two possible metabolic pathways with certainty.

Transamination. Brain and liver tissue extracts catalyse a transamination reaction between γAB and αKG (Bessman *et al.*, 1953; Roberts and Bregoff, 1953). A similar transaminase has been demonstrated in a number of microorganisms, e.g. *Aspergillus fumigatus* (Roberts, Ayengar, and Posner, 1953), *Escherichia coli* (Roberts, 1954), *Endomycopsis vernalis* (Kating, 1954), *Achromobacter* (Higashi *et al.*, 1957), *Pseudomonas aeruginosa* (Noe and Nickerson, 1958; Bachrach, 1960), *Pseudomonas fluorescens* (Scott and Jakoby, 1959), and *Bacillus pumilis* (Tsunoda and Shiio, 1959). The relative ease with which a γAB-αKG transaminase has been demonstrated in animal and microbial systems is in striking contrast to the situation encountered in higher plants, where reliable demonstrations of the enzyme system are extremely rare (see Part 2).

Yeast transaminase. An acetone powder preparation from cells of *Torulopsis utilis* was used as a source of transaminase enzyme. The acetone powder was extracted with 0·1 M. phosphate buffer of appropriate pH (2 ml. were used for each gramme fresh weight of original yeast cells) and, after removal of insoluble matter by centrifugation, the supernatant was dialysed overnight against more 0·1 M. phosphate buffer at 4° to yield the crude transaminase preparation. The standard assay system contained the following substances present at the final concentrations stated: L-amino acid (0·05 M.), keto acid (0·05 M.), pyridoxal phosphate (8×10^{-5} M.), and transaminase preparation (0·5 ml./ml.); reactions were performed at 37°. The course of the reactions was followed by assaying the amount of amino-acid product using quantitative paper-chromatographic methods (based on Fowden, 1951). Alternatively, in the special case where αKG was the acceptor keto acid, glutamate produced was determined manometrically using an acetone-powder preparation of *Escherichia coli* as a source of glutamic decarboxylase.

The rates at which various amino-acids donated their amino group to αKG are given in Table 1 (Expt. 1). When the crude enzyme preparation was obtained from *Torulopsis* cells grown on γAB-medium, the rate of transamination (per mg. protein-N present in the enzyme preparation) measured with γAB was far higher than that determined using cells grown on NH_4^+-medium. The transamination rates measured for other amino-acids were affected only slightly by the two different types of N-nutrition used to produce the *Torulopsis*. Other workers have reported that γAB nutrition leads to an increased synthesis of a γAB transaminase in other organisms, e.g. *Aspergillus fumigatus*

(Roberts *et al.*, 1953) and *Pseudomonas* species (Scott and Jakoby, 1959; Noe and Nickerson, 1958). These data provide indirect support for the existence in *Torulopsis* of a transaminase enzyme specific for γAB as the amino-group donor.

TABLE 1

Showing the Relative Rates of Transamination of Various Amino-acids with α-Ketoglutarate Catalysed by Acetone-Powder Extracts of Torulopsis utilis

(The rates observed for various amino-acids are expressed as percentages of the rate observed when aspartic acid was the amino-group donor)

Amino-acid substrate	Relative amounts of glutamate formed using: Extract of γAB-grown yeast	Extract of NH_4^+-grown yeast
Experiment 1:		
γAB	60	19
Isoleucine	91	111
Valine	103	124
Alanine	24	24
Arginine	3·7	4·4
Aspartic acid	100	100
Experiment 2:		
γAB	63	7·7
β-Alanine	10	7·1
ε-Aminocaproic acid	2·9	4·7
γ-Aminovaleric acid	0·5	1·1
Lysine	12	17
Aspartic acid	100	100

TABLE 2

Showing the Rates of Transamination of α-Ketoglutaric Acid with Various Amino-acids used Singly and in Combination with γAB

(Results are expressed as μg. α-NH_2-N introduced into glutamate per ml. of standard assay system per hour)

Amino-acid substrate	Glutamic acid formed: (i) αKG + single amino-acid	(ii) αKG + γAB + single amino-acid	Difference (ii)–(i)
γAB	29·0*	—	—
Aspartic acid	74·4	101·0	26·6
Alanine	6·2	34·8	28·6
Isoleucine	50·8	81·6	30·8
Valine	32·9	59·0	26·1
Glycine	3·0	34·1	31·1

* Value is mean of four separate determinations.

Similar results were obtained in another experiment (Table 1, Expt. 2) where the rates of transamination of various non-α-amino-acids with αKG were compared with the rates obtained with γAB as substrate. Again, the different conditions of N-nutrition did not alter significantly the transamination rates determined for these amino-acids except in the instance of γAB.

Further support for the idea that a distinct enzyme catalyses the transamination of γAB was provided by the results of experiments in which the rates of

glutamate formation from αKG were measured when a mixture of γAB and one other amino-acid acted as the amino-group donor. Table 2 shows that no competition between amino-acid substrates occurred; the amount of transamination measured with mixtures of two amino-acids was equal to the sum of the individual rates determined when the acids were used singly.

The transamination reaction between γAB and αKG was easily reversed, and had an equilibrium constant:

$$K = \frac{[\text{SSA}]\,[\text{glutamate}]}{[\gamma\text{AB}]\,[\alpha\text{KG}]} = 0{\cdot}12.$$

The reaction was studied in the reverse direction, using SSA prepared by a modification of the method of Dakin (1917) and purified by an adaptation of the procedure of Carrière (1922).

No requirement for pyridoxal phosphate as coenzyme could be demonstrated even when enzyme preparations that had been subjected to very prolonged dialysis were used.

The present conclusions concerning the specificity, equilibrium constant, and coenzyme requirement of the γAB-αKG transaminase reaction in *Torulopsis* are closely similar to those reached by Scott and Jakoby (1959), who studied this reaction using enzyme from *Pseudomonas fluorescens*.

The Michaelis' constant for the reaction catalysed by the yeast enzyme was determined by measuring the rate of transamination at various γAB concentrations within the range 0·005–0·05 M. (αKG concentration was kept constant at 0·05 M.); a value of 4×10^{-3} M. was obtained. The optimum pH determined was approximately 8·2, but variation of enzyme activity with pH was not very pronounced. These values for Michaelis' constant and optimum pH are in excellent agreement with those of 3×10^{-3} M. and 8·2 respectively determined for a similar transaminase prepared from cattle brain tissue (Baxter and Roberts, 1958).

E. *Related Inductive Increases of Respiratory and Transaminase Activities*

The gradual increase occurring in respiratory rate (O_2 uptake) of NH_4^+-grown *Torulopsis* cells after they had been supplied γAB (see Section 1C above) suggested that at least one enzyme involved in the early stages of γAB degradation was adaptive in character. The results presented in Section 1D clearly indicate that this enzyme may be a γAB transaminase. Therefore, increases of O_2 uptake and transaminase activity were determined simultaneously to test the degree of correlation existing between the two activities.

About 20 g. fresh weight of *Torulopsis utilis*, originally grown on NH_4^+-medium, were shaken in N-free medium for about 1 hour. After centrifugation, the cells were transferred to about 300 ml. of 0·15 M. phosphate buffer, pH 6·5. Small portions of the cell suspension were introduced in manometric flasks, and the rate of O_2 uptake was measured at 25°; the final reaction mixture contained 0·1 M. phosphate buffer, 0·09 M. γAB, and 15·5 mg. dry-weight yeast cells/ml. Occasional aeration of the manometric flasks was

necessary to restore their oxygen levels at times marked 'A' in Fig. 3, which illustrates the time-course of O_2 uptake. γAB was added to four larger portions of yeast suspension (maintained at 25° with continuous shaking) to give the same final concentrations of reactants as those present in the manometric flasks. At the times indicated on Fig. 3 (S_0, S_1, S_2, and S_3), a portion of yeast

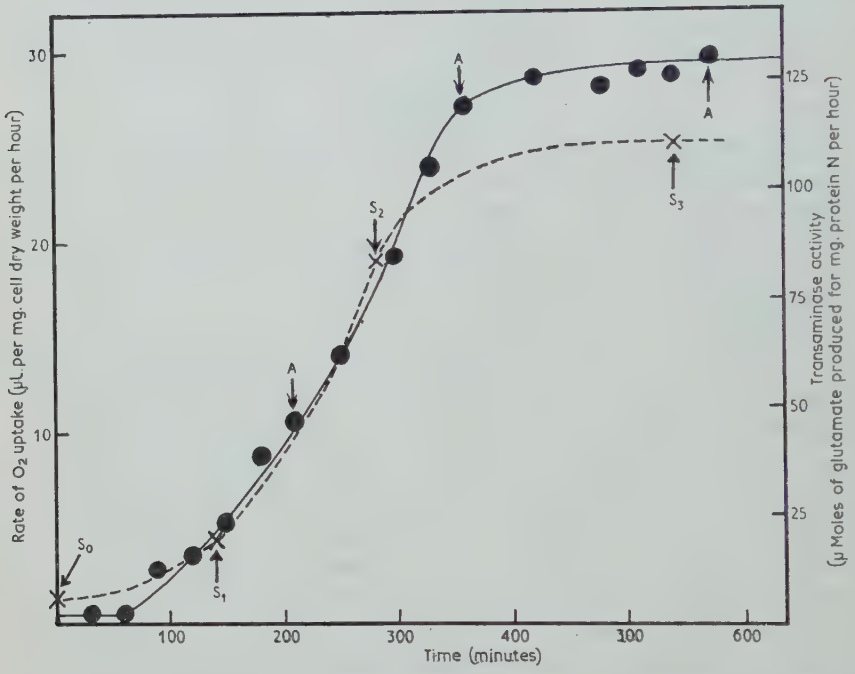


FIG. 3. Illustrates the relationship existing between the rates of O_2 uptake at 25° of intact cells of *Torulopsis utilis* at pH 6·5 in the presence of 0·09 M. γAB and the amounts of γAB-αKG transaminase present within the cells at various times (S_0, S_1, S_2, S_3) after they were supplied γAB. ●—●, rate of O_2 uptake; x—x, transaminase activity.

was harvested by centrifugation and, after washing with a weak solution of γAB, was converted to an acetone powder. The γAB-αKG transaminase activity present in the four acetone powders obtained was measured using the standard assay conditions described in Section ID. The values obtained for respiratory rates and transaminase contents are illustrated in Fig. 3. The results support the view that the rate of O_2 uptake associated with γAB metabolism in *Torulopsis* is controlled by the level of a γAB transaminase enzyme; the increases in transaminase activity measured at S_1, S_2, and S_3 (above that of unadapted cells at S_0) were associated with similar proportionate increases in the rates of O_2 uptake determined at these times.

2. Metabolism of Succinic Semi-aldehyde

Although SSA has been established as a product of transamination when γAB reacts with αKG in the presence of *Torulopsis*, brain (Bessman *et al.*,

1953), and *Pseudomonas fluorescens* (Scott and Jakoby, 1959) extracts, the aldehyde has never been proved conclusively to be a normal constituent of plant tissues. The normal analytical method employed for the detection of aldehyde and keto acids depends upon their conversion to yellow-coloured 2:4-dinitrophenylhydrazones. These derivatives may be identified by direct, visual, paper-chromatographic methods or they may be converted by catalytic hydrogenation into their analogous amino-acids which can then be characterized by paper chromatography. The derivative of SSA would yield γAB upon hydrogenation. However, hydrogenation of the derivative of αKG (a ubiquitous plant constituent) produces not only glutamic acid, but considerable amounts of γAB (Towers, Thompson, and Steward, 1954). Therefore, whilst the latter workers have shown that γAB was produced when the mixed 2:4-dinitrophenylhydrazones of keto acids derived from several plants were hydrogenated, the γAB may be merely an artefact having no significance in proving the presence of SSA in the original plant materials. No SSA could be detected in *Torulopsis* or *Saccharomyces* by direct chromatography of the 2:4-dinitrophenylhydrazones of the keto acids extracted from the cells, even when the yeasts were grown on γAB-medium. Presumably the molecules of SSA formed by transamination of γAB are metabolized swiftly by other reactions and so the aldehyde is not accumulated to levels capable of detection by existing chromatographic methods.

A. *Oxidation of SSA*

Direct oxygen uptake. Preliminary experiments established that both *Torulopsis* and *Saccharomyces* could utilize SSA as a respiratory substrate. Animal tissues have been shown to catalyse a similar oxidative degradation of SSA (Sugiura, 1957; Tsukada, Nagada, and Takagaki, 1957). Unlike the earlier results obtained using γAB (see Figs. 2 and 3), the rate of O_2 uptake associated with SSA metabolism by whole *Torulopsis* cells showed only a small increase with time.

In later experiments the rates of SSA and succinate metabolism were compared under various conditions. Di- and tri-carboxylic acids penetrate into cells of baker's yeast only very slowly, but the permeability barriers may be largely overcome by exposing the cells to solid CO_2 (Krebs, Gurin, and Eggleston, 1952). This method was not very effective when applied to *Torulopsis* cells, and *Saccharomyces* has been used in most of the subsequent experiments. The treatment with solid CO_2 was shown not to affect the rate of oxidation of SSA. The oxidative metabolism of SSA and succinate also has been studied, using cell-free homogenates of *Saccharomyces* obtained by breaking the cells in 0·15 M. phosphate buffer using a Nossal (1953) shaker, and with mitochondria prepared by the method of Linnane and Still (1955). In experiments with homogenates or mitochondria, maximum rates of O_2 uptake could be obtained by adding a range of cofactors to the reaction mixture to give the following final concentrations: TPN, 5×10^{-5} M.; DPN, 5×10^{-5} M.; cytochrome c, 2×10^{-5} M.; ATP, 5×10^{-4} M.; Mg^{++}, 0·007 M.

The rate of O_2 uptake associated with the metabolism of 0·03 M. succinate was higher than that obtained with 0·03 M. SSA using all three yeast preparations; the difference of rates was smallest when mitochondria were used (see Table 3). When 0·03 M. malonate was added to the reaction systems, the inhibition of succinate metabolism was more pronounced than that of SSA. The extent of the inhibition of SSA oxidation by malonate appeared to be related to the degree of enzymic organization existing in the reaction system, and inhibition was most marked when highly organized mitochondria were

TABLE 3

Showing the Relative Rates of O_2 uptake when Succinate and Succinic Semi-aldehyde were Metabolized by Various Preparations from Saccharomyces cerevisiae, *and the Effect of Additions of Malonate*

(O_2 uptake expressed as μl. per hour)

	Oxygen uptake attributable to metabolism of substrate		
	With CO_2-treated cells	With cell-free homogenate	With mitochondria
0·03 M. Succinate	81	123	144
0·03 M. Succinic semi-aldehyde	27	55	128
0·03 M. Succinate + 0·03 M. Malonate	9 (11%)	5 (4%)	40 (27%)
0·03 M. Succinic semi-aldehyde + 0·03 M. Malonate	14 (52%)	42 (76%)	45 (37%)

Figures in parentheses represent percentages of uninhibited oxidative activity remaining in the presence of malonate.

used and lowest in cell-free homogenates. If SSA is first converted to succinate (see later), then in mitochondria the latter acid will enter the organized tricarboxylic acid cycle reaction sequence; therefore SSA will undergo an extensive degradation that involves several oxidative reactions. When cell-free homogenates were used the coupled, multi-enzyme system involved in succinate metabolism would be disintegrated and further metabolism of succinate would be restricted. Therefore, when mitochondria were used, a large proportion of the total O_2 uptake associated with SSA metabolism was probably due to the further oxidation of succinate; with cell-free homogenates O_2 uptake could be attributed largely to the primary production of succinate from SSA. Against this theoretical background, the results of Table 3 indicate that malonate has little, if any, inhibitory effect upon this primary reaction.

The rate of O_2 uptake was found to increase with increasing SSA concentrations up to at least 0·05 M.; this finding is in sharp contrast to the results obtained in the later experiments where the spectrophotometric reduction of DPN by SSA was studied.

Succinic acid was identified as a product of SSA oxidation. 0·01 M. SSA was used as substrate for the mitochondrial preparation obtained from *Saccharomyces* and 70 per cent. of the initial aldehyde had been metabolized in 3 hours. The remaining SSA was largely precipitated as its 2:4-dinitrophenyl-

hydrazone, and after centrifugation the supernatant was passed through an anion-exchange resin column of Amberlite IRA 400. Unchanged 2:4-dinitrophenylhydrazone and any soluble derivatives were absorbed strongly by the resin together with organic acids. The latter were eluted with N.$(NH_4)_2CO_3$ solution but all the yellow-coloured substances were retained by the resin. Succinic acid was detected in portions of the eluate run on paper chromatograms developed in the solvent of Bentley (1952). Succinic acid was not detected when this extraction procedure was applied to a control reaction mixture of mitochondria and cofactors without SSA. Previously succinic acid has been reported as a product of the oxidation of SSA by intact cells of *Mycobacterium avium* (Shoji, Mori, and Ito, 1958).

Although intact *Torulopsis* cells are capable of oxidizing SSA, the results obtained using cell-free extracts and mitochondrial preparations from *Torulopsis* were erratic; the rates of O_2 uptake in the presence of SSA were sometimes larger than, and at other times less than, the endogenous rates. Two explanations appeared possible. The first assumed that endogenous respiration was inhibited by the SSA concentration used; however, lower SSA concentrations gave similar results and so this idea seemed untenable. Alternatively, in *Torulopsis* extracts, an enzymic reduction of SSA may be superimposed upon the oxidative metabolic pathway (see Section 2B).

Succinic semialdehyde dehydrogenase. Partially purified preparations of this enzyme, capable of catalysing a reaction in which oxidation of SSA was coupled to the reduction of either DPN or TPN, have been obtained from animal tissue (Albers and Salvador, 1958), from *Pseudomonas fluorescens* (Jakoby and Scott, 1959), and from another species of *Pseudomonas* (Nirenberg and Jakoby, 1960). Black (1951, 1955) and Seigmiller (1953) have obtained aldehyde dehydrogenases from baker's yeast. The enzyme preparation obtained by Black was DPN-linked and K^+-activated. Dehydrogenase activity was highest for acetaldehyde but other aldehyde substrates were reduced at slower rates, e.g. SSA at 24 per cent. of the rate measured for acetaldehyde. Seigmiller's enzyme was TPN-linked and Mg^{++}-activated.

The aldehyde dehydrogenase activity of *Saccharomyces* has been re-examined in our experiments particularly in respect to SSA as substrate. The enzyme preparation used was that obtained after stage 3 of Black's (1955) purification procedure. At this stage the preparation would be expected to contain both the DPN- and TPN-linked enzymes. The enzyme preparation was dissolved in weak phosphate (0·025 M.) solution of pH 6·3 and stored at −20° until required.

Dehydrogenase activity was assayed by estimating the reduction of DPN or TPN spectrophotometrically at 340 mμ. In addition to enzyme preparation, the standard assay mixture contained the following substances at final concentrations stated: 0·1 M. tris buffer, pH 8·0; 0·1 M.KCl; 5×10^{-4} M.DPN; glutathione, 1×10^{-3} M. Glutathione was added because both aldehyde dehydrogenases are reported to be dependent upon free —SH groups for their action (Stoppani and Milstein, 1957). SSA or acetaldehyde were added as

substrates and final concentrations used were normally 0·00102 M. The main properties of the enzyme are summarized below.

Optimum substrate concentration. Substrate inhibition was observed at very low SSA concentrations (see Fig. 4). At the lowest concentration used (0·00034 M.), the initial reaction rate was highest although the rate of DPN

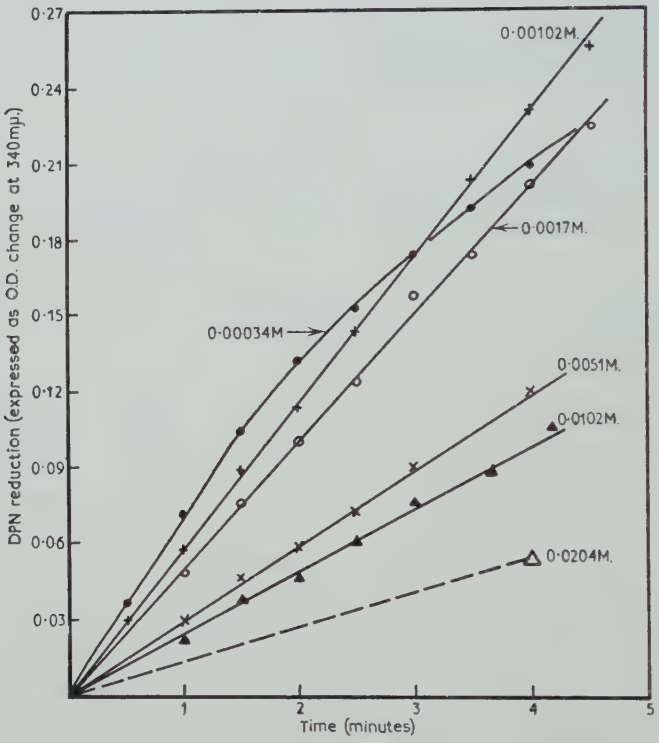


FIG. 4. Illustrates the effect of substrate concentration upon the rate of dehydrogenation of SSA by a partially purified SSA dehydrogenase from baker's yeast.

reduction fell appreciably with time during a 5-minute reaction period. The concentration of SSA present in the standard assay system gave the highest linear reaction rate.

The relative rates at which SSA and acetaldehyde were oxidized varied for different batches of enzyme preparation. Extreme cases observed included a preparation which oxidized acetaldehyde twelve times as rapidly as SSA and another capable of oxidizing SSA nearly three times faster than acetaldehyde.

Optimum pH. The rate of SSA oxidation showed a rather sharp peak of activity at pH 9·2. Under the same conditions, acetaldehyde oxidation proceded at a nearly constant rate between pH 8·6 and 9·2, with an indistinct maximum activity at about pH 9·0. Jakoby and Scott (1959) report maximal activity at pH 8·5 for the SSA dehydrogenase from *Pseudomonas fluorescens*. More recently, Nirenberg and Jakoby (1960) have resolved the SSA dehydro-

genase activity present in another *Pseudomonas* species into two distinct enzyme components; one is dependent upon TPN and has a pH optimum of about 9·2, whilst the other is DPN-linked with pH optimum of 8·0.

Cofactor requirement. The rate of SSA dehydrogenation in the standard assay system was compared with the rate when TPN replaced DPN and when Mg^{++} ions were added. The rate of reaction with DPN was approximately four times that observed with TPN. Mg^{++} ions had no effect upon the reaction rate when DPN was used, but if Mg^{++} ions were omitted from the TPN-linked system the rate was reduced 2–5-fold. The SSA dehydrogenase

TABLE 4

Showing the Differing Degrees of Temperature Lability of the Aldehyde Dehydrogenase Activity from Saccharomyces cerevisiae *in respect of two Substrates, Succinic Semialdehyde and Acetaldehyde*

(Dehydrogenase activities are expressed as percentages of that measured for each substrate using an untreated enzyme preparation)

Enzyme preparation heated for 5 minutes at:	Activity with SSA as substrate	Activity with acetaldehyde as substrate
70°	0	0
60°	0	58
55°	0	98
50°	62	—
no heat treatment	100	100

of *P. fluorescens* (Jakoby and Scott, 1959) could utilize both TPN and DPN, although the former was 8·2 times more active under the assay conditions used, which did not include Mg^{++} ions. Another *Pseudomonas* sp. contained distinct DPN- and TPN-linked SSA dehydrogenases (Nirenberg and Jakoby, 1960). The former could reduce TPN at one-twelfth the rate of DPN; similarly, the TPN-linked enzyme could also reduce DPN, but again at only one-twelfth the rate of TPN.

Effect of NaCl. Black (1951) observed that Na^{+} ions inhibited acetaldehyde oxidation by his DPN-linked, K^{+}-activated aldehyde dehydrogenase. When 0·2 M. NaCl was added to our standard assay system, the rate of oxidation of SSA was reduced by 25 per cent. and that of acetaldehyde by 50 per cent.

Glyoxylate inhibition. Two semialdehydes, glyoxylate and malonic semialdehyde, act as competitive inhibitors of SSA dehydrogenase (Jakoby and Scott, 1959). In our assay system with yeast enzyme, addition of 0·00102 M. and 0·00204 M. glyoxylate produced a 25 per cent. and 40 per cent. reduction of dehydrogenase activity when SSA was the substrate. Glyoxylate caused no inhibition of acetaldehyde oxidation.

Heat denaturation. The yeast enzyme preparation was heated for 5 minutes at temperatures of 50°, 55°, 60°, and 70° and then the residual dehydrogenase activity against SSA and acetaldehyde was assayed. Table 4 presents the results obtained. The DPN-linked, SSA dehydrogenase activity was destroyed completely at a temperature of 55° (compare Nirenberg and Jakoby,

1960), whereas acetaldehyde dehydrogenase activity was largely unaffected by this treatment.

These properties indicate that the yeast preparation probably contained two SSA dehydrogenases. The dehydrogenase utilizing DPN was more active than the TPN-linked enzyme and was chosen for more detailed study. In many respects this enzyme was like the acetaldehyde dehydrogenase of Black (1951), but there seems little doubt that dehydrogenation of these two aldehydes was catalysed by two distinct DPN-linked enzymes which were both present in the yeast enzyme preparation. It is impossible to decide at present whether the TPN-linked SSA dehydrogenase of yeast is identical with the two TPN-linked dehydrogenases of *Pseudomonas* species (Jakoby and Scott, 1959; Nirenberg and Jakoby, 1960), for, although the latter enzymes utilized TPN preferentially, it remains uncertain whether Mg^{++} ions are necessary for their activation.

B. *Reduction of SSA*

Acetaldehyde is reduced to ethyl alcohol in a reaction catalysed by alcohol dehydrogenase. The erratic results obtained in experiments when the O_2 uptake associated with SSA metabolism was measured using various preparations from *Torulopsis* (the ease with which O_2 uptake was demonstrated with intact cells contrasted sharply with our inability to obtain O_2 uptake using cell-free extracts, see Section 2A) prompted a search for a similar enzyme capable of catalysing a reduction of SSA to its corresponding alcohol, γ-hydroxybutyric acid (γOHB). Both the mitochondrial and microsomal plus soluble (supernatant) fractions obtained from broken *Torulopsis* cells by fractional centrifugation catalysed an endogenous reduction of TPN; in the presence of SSA, the apparent rate of TPN reduction was decreased markedly. When SSA was added to the supernatant preparation (the composition of the complete reaction mixture is given in the caption to Fig. 5) after a 3-hour period during which TPN reduction had proceeded, a rapid reoxidation of TPNH occurred (Fig. 5, upper line).

γOHB was detected as the product of SSA reduction by the following procedure. The reaction mixture was first acidified; in this way, γOHB was converted into butyrolactone, which was removed from the mixture by steam distillation. The distillate was refluxed with dilute potassium hydroxide solution for 30 minutes to obtain the potassium salt of γOHB, excess alkali being neutralized by careful titration with dilute H_2SO_4. After evaporation, the solid residue of the potassium salt of γOHB, admixed with some K_2SO_4, was treated by a modification of the methods used for the preparation of hydroxamic acids described by Fink and Fink (1949) and Thompson (1950). The hydroxamate of γOHB present in the reaction mixture was obtained in this way, and this was shown to be identical with an authentic sample of γOHB hydroxamate by paper chromatography using an *n*-butanol–acetic acid–water mixture (Partridge, 1948) as solvent. The chromatogram was developed by spraying with a solution of $FeCl_3$ in *n*-butanol (Fink and Fink, 1949). The

identity of the reaction product was established also by chromatography of Fe^{+++} complexes of the hydroxamates (Bayer and Reuther, 1956), and by the conversion of butyrolactone (obtained after steam distillation) first into 2-pyrrolidone by treatment with anhydrous ammonia (Späth and Lintner, 1936) and subsequently into γAB after alkaline hydrolysis of the pyrrolidone (Tafel and Stern, 1900). γOHB was not present in reaction systems from which SSA was omitted.

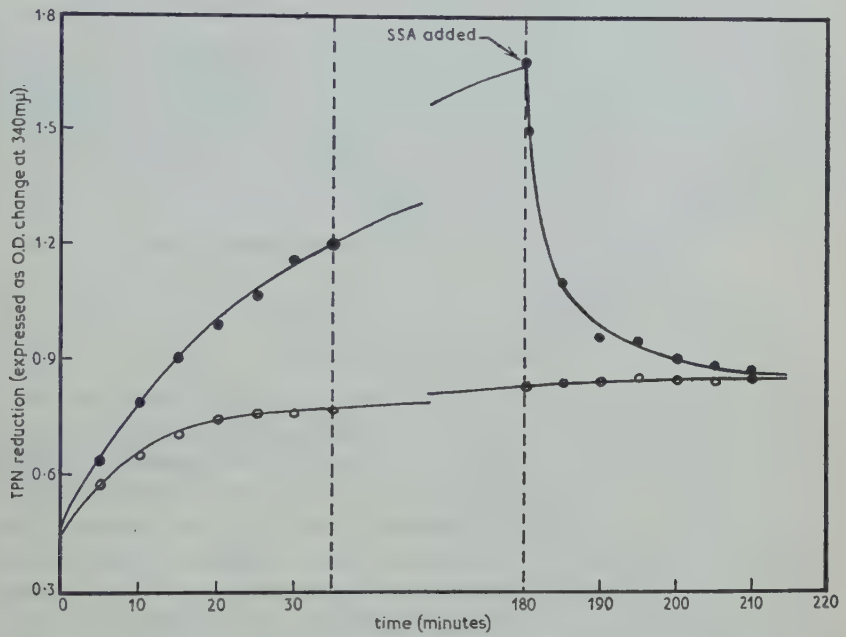


FIG. 5. Illustrates the reduction of TPN by a non-particulate extract of *Torulopsis utilis*, and the effect of added SSA upon the rate of reduction. ●—●, no SSA added initially; ○—○, with SSA added initially.

Properties of γOHB dehydrogenase. An enzyme catalysing the reversible interconversion of SSA and γOHB has been isolated from a *Pseudomonas* species (Nirenberg and Jakoby, 1960) and has been designated γOHB dehydrogenase. The enzyme present in extracts of *Torulopsis* cells grown on γAB-medium also catalysed the reverse reaction in which γOHB was oxidized. The equilibrium was in favour of γOHB formation and reversibility could be demonstrated only when high concentrations of γOHB (0·1 M.) were used. In the usual assay system where TPN was the coenzyme, the product of γOHB oxidation was shown to be SSA (identified by paper chromatographic analysis of 2:4-dinitrophenylhydrazones).

The yeast enzyme appeared to be TPN-linked and DPNH was inactive as a cofactor for SSA reduction. When γOHB was the substrate, DPN was reduced at approximately the same rate as TPN at pH 6·0. However, whereas SSA was identified as the reaction product when TPN was used, no SSA

could be detected when DPN was reduced. This last observation, whilst of a preliminary nature, indicated that another pathway for γOHB metabolism may exist in *Torulopsis*. A completely different pyridine nucleotide specificity was demonstrated for the γOHB dehydrogenase of *Pseudomonas* (Nirenberg and Jakoby, 1960); this enzyme utilized DPN far more efficiently than TPN.

The optimum pH for the reduction of SSA by TPNH was about 5·8 when catalysed by the dehydrogenase from *Torulopsis*. Nirenberg and Jakoby (1960) report an optimum pH of 7·0 for the DPNH-linked reduction of SSA by the *Pseudomonas* enzyme.

The facts presented show that both *Torulopsis* and *Pseudomonas* cells contain a γOHB dehydrogenase which catalyses the reversible interconversion of SSA and γOHB. However, little doubt can exist that the two organisms contain distinct enzymes that differ considerably in their properties. The reactions catalysed by extracts of *Torulopsis* may be summarized as:

$$\text{SSA} \underset{\text{TPNH}}{\overset{\text{TPN}}{\rightleftharpoons}} \gamma\text{OHB} \xrightarrow{\text{DPNH}} \text{unknown product?}$$

3. The Initial Steps of the Metabolic By-pass

A. *Glutamic dehydrogenase*

Glutamic dehydrogenase is widely distributed and the properties of the enzyme present in yeast (presumably *Saccharomyces cerevisiae*) have been described by Adler, Gunther, and Everett (1938). The presence of glutamic dehydrogenase in cell-free, dialysed extracts of *Torulopsis utilis* (disintegrated by the method of Nossal, 1953) was established by observing the spectrophotometric reduction of DPN at 340 mμ. The reaction was performed in 0·1 M. phosphate buffer, pH 7·6, with final concentrations of glutamic acid, 0·02 M., and DPN, 3×10^{-4} M. After equilibrium had been attained, the reversibility of the reaction was established by addition of αKG which rapidly re-oxidized some of the DPNH originally produced.

B. *Glutamic decarboxylase*

Glutamic decarboxylase activity occurs in the red yeast, *Rhodotorula glutinis* (Krishnaswamy and Giri, 1956).

Decarboxylase activity has now been detected in *Saccharomyces* extracts. *Saccharomyces* cells were shaken overnight in the N-free medium and then disintegrated in 0·02 M. phosphate solution by the method of Nossal (1953). The cell-free extract obtained after centrifuging was diluted with two volumes of 0·5 M. acetate buffer, pH 4·5, and used as a source of decarboxylase activity. Ten μmoles of glutamic acid were added to manometric flasks containing 3 ml. of the diluted yeast extract; CO_2 equivalent to decarboxylation of 40 per cent. of the glutamate supplied was evolved in 1·5 hours. γAB was detected as the product of decarboxylation.

Glutamic decarboxylase activity could not be demonstrated unequivocally in yeast cells that had not been N-starved. In such cells, high levels of endo-

genous glutamic acid presumably existed and extracts from them showed rates of endogenous CO_2 production that were not increased on addition of extra glutamate.

Discussion

γAB is one of many amino-acids recognized as plant constituents since the introduction of paper-chromatographic techniques (for reviews, see Fowden, 1958, 1960). Like the majority of these newly characterized substances, γAB does not appear to be a constituent of proteins, although apparently it is present in a bound form in acid-hydrolysable conjugates present in root nodules of *Trifolium* (Butler and Bathurst, 1958). The reactions of γAB now established for yeasts and *Pseudomonas* confer a definite metabolic role upon the amino-acid.

All the enzymes implicated in the by-pass reaction scheme outlined in the Introduction have been shown to be present in *Saccharomyces*, whilst in *Torulopsis* only glutamic decarboxylase has not been established with certainty. Metabolism of αKG via the by-pass mechanism is therefore possible, but the present results do not provide any indication of the percentage of αKG molecules that traverse this metabolic pathway. Indeed, it is difficult to devise an experiment, radioisotopic or otherwise, that could yield a definite answer concerning the relative *in vivo* importances of the alternative mechanisms for converting αKG into succinate. The importance of the by-pass reactions obviously would be enhanced under conditions where the operation of the tricarboxylic acid cycle was inhibited at the αKG stage. Therefore it is interesting to note that such situations have been reported. Oxidative decarboxylation of αKG in animal tissues was inhibited by the lactone form of γ-methyl-γ-hydroxy-α-ketogluturate, a dimer of pyruvate present in old preparations (Montgomery and Webb, 1954); of even greater interest is the fact that αKG metabolism is partially blocked in *Bryophyllum* apparently because this plant accumulates large quantities of pyruvate, which give rise to the production of the dimer (Ramstad and Lieberman, 1954).

The metabolic significance of the reduction of SSA to γOHB by *Torulopsis* extracts is unknown. The suggestion may be advanced that an isomerase (or a group of enzymes) effects a transfer of the hydroxyl group to the β-carbon atom. The β-hydroxybutyrate produced then would be available to participate in the reactions involved in fatty acid metabolism.

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γ-Aminobutyric Acid Metabolism in Plants

Part 2. Metabolism in Higher Plants

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With five Figures in the Text

ABSTRACT

The metabolism of γ-aminobutyric acid (γAB) has been studied in higher plants, particularly in peas and peanuts. Transamination appeared to form the first step in γAB degradation although transaminase activities were very low. The relatively active γAB transaminase associated with whole pea plants possessing nodulated roots appears to reside almost entirely within the nodules. γAB transamination was demonstrated conclusively in extracts of mitochondria from cotyledons of peanut seedlings; pyruvic acid acted as a better amino-group acceptor than α-ketoglutaric acid (αKG). γAB transaminase activity present in the microsomal and soluble cytoplasmic fractions of the cells was very low.

γAB was not metabolized perceptibly by intact mitochondria from peanut, but when various organic acids were supplied simultaneously, an extra uptake of oxygen occurred and was associated with γAB disappearance. Aspartate, alanine, and ammonia were formed using the nitrogen atom of γAB.

The metabolic pathway followed by the carbon skeleton of γAB was traced by supplying C^{14}-labelled material to leaf discs of peas and to mitochondria from peanut cotyledons. Radioactivity was incorporated into organic acids, amino-acids, and respiratory carbon dioxide in a manner suggesting that γAB was converted into succinate which was then metabolized by the enzymes of the Krebs cycle present in the plant mitochondria.

Glutamic decarboxylase was shown to be present largely in the non-particulate (soluble) cytoplasm of cells. The enzymes responsible for γAB synthesis and degradation, glutamic decarboxylase, and γAB transaminase, respectively, therefore largely reside in different sub-cellular fractions.

INTRODUCTION

PROBABLY γ-aminobutyric acid (γAB) is present in all higher plants. Certainly glutamic acid (GA) is universally distributed in plants and is nearly universally accepted as the metabolic precursor of γAB. The distribution of GA decarboxylase, the enzyme associated with this conversion, has been the subject of several painstaking surveys and numerous plant species possess marked decarboxylase activity (Schales, Mims, and Schales, 1946; Kulkarni and Sohonie, 1956; Zaragoza, 1959). The formation of C^{14}-γAB from C^{14}-GA in barley was favoured by anaerobic, dark conditions, presumably because simultaneous oxidation of GA was suppressed (Naylor and Tolbert, 1956).

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In contrast, the information at present available concerning the pathway of γAB degradation is sparse, although the older tendency to regard the amino-acid as an end-product of nitrogen metabolism is no longer tenable. The rejection of this concept was inevitable when large proportions of the C^{14} atoms supplied initially as C^{14}-labelled γAB to carrot root (Steward, Bidwell, and Yemm, 1958) and *Convallaria* leaves (Fowden and Bryant, 1959) were shown to be present in other tissue constituents and the respiratory CO_2 after 24 hours. The results of Naylor and Tolbert (1956) suggest that an oxidative reaction must be involved at an early stage in γAB degradation in barley, although this need not be the primary reaction (compare the findings for *Torulopsis* in Part I). The results contained in the present paper suggest that the deamination of γAB in higher plants again may be ascribed largely to the action of transaminases. They also established that the carbon skeleton resulting was converted largely to CO_2 after participating in intermediary reactions involved in the tricarboxylic acid cycle.

Materials and Methods

Plant materials. Peas (*Pisum sativum* var. Little Marvel) were grown in vermiculite, sterilized by autoclaving, in normal daylight at 20° in a greenhouse. They were harvested 3 weeks after sowing. Mung beans (*Phaseolus aureus*) and peanuts (*Arachis hypogaea*) were grown in vermiculite at 33° in the dark for 2 and 5 days respectively.

Rhizobium was isolated from pea root nodules and grown on yeast mannitol agar: mannitol 10 g., K_2HPO_4 0·5 g., $MgSO_4.7H_2O$ 0·2 g., $CaCO_3$ 3·0 g., yeast extract 100 ml., and water 900 ml. *Escherichia coli* (N.C.T.C. 8584) was grown on Lemco broth: Lemco 10 g., NaCl 5 g., peptone 10 g., glucose 20 g., and water to 1 l. The pH was adjusted to 6·0.

When nodulated pea plants were required, the seedlings were infected with a culture of the isolated *Rhizobium.*

Preparation of sub-cellular particulate fractions. Mitochondrial fractions were isolated from homogenates of seedlings prepared by the method of Bonner and Millerd (1953) by centrifugation at 20,000 *g.* for 25 minutes. A further particulate fraction, hereafter termed microsomes, was prepared by centrifuging the supernatant from the mitochondrial preparation at 90,000 *g.* for 30 minutes. The resulting microsomal pellet was suspended in a solution containing 0·2 M. KCl and 0·05 M. phosphate buffer, pH 7·4, and centrifuged again at 90,000 *g.* for 30 minutes. The temperature throughout these operations was kept below 4°.

Preparation of C^{14}-γAB. Labelled γAB was prepared by decarboxylation of uniformly labelled C^{14}-glutamate using an acetone-powder of *E. coli.* The γAB was isolated by absorption on a Zeocarb 215 resin column and separated from radioactive impurities on a paper chromatogram developed with an *n*-butanol–acetic acid–water mixture (Partridge, 1948). The C^{14}-γAB eluted from the paper had a specific activity of 10 mC. per m.mole.

1. Transamination of γAB

As yet no convincing demonstration has been obtained of the presence of a γAB-αKG transaminase in higher plants. Miettinen and Virtanen (1953) found γAB-αKG transaminase to be present in well-nodulated roots of peas, but the possibility remained that the transaminase may be confined to the nodules and be associated only with the *Rhizobium*. Wilson, King, and Burris (1954) demonstrated a small production of C^{14}-labelled GA from γAB and C^{14}-labelled αKG in a survey to ascertain which amino-acids could transaminate with αKG in the presence of a lupin seedling extract. The specific activity of the GA produced when γAB was used was only slightly higher than that of GA obtained in a control experiment when no amino-acid was supplied and therefore it is doubtful whether this small difference can be accepted as real evidence in confirmation of γAB-αKG transaminase. Suzuki *et al.* (1958) found that GA was produced from γAB and αKG using a preparation from potato tubers. The amount obtained represented only about 1 per cent. of the substrate added, and the experimental conditions (24 hours' incubation at 37°) were such that microbial contamination almost certainly occurred: confirmation of these results is therefore necessary.

Reports of failure to find γAB-αKG transaminase in plants have been published. Roberts and Bregoff (1953) failed to find the transaminase in extracts of squash and pepper fruits using techniques which demonstrated the enzyme in brain tissue. Rijven (1960) failed to demonstrate the transaminase in wheat seedlings even after concentration of protein by ammonium sulphate precipitation.

γAB-αKG transaminase has been found to be the primary step in the pathway of γAB degradation in animals and micro-organisms. Therefore it is important to establish with certainty whether this transaminase does occur in higher plants. The results obtained in experiments to be described definitely confirm the presence of the enzyme in certain higher plants, although only low activities were demonstrated. Evidence is given to indicate that the transamination reaction involving γAB observed by Miettinen and Virtanen (1953) probably was due entirely to the action of the microbial symbiont, *Rhizobium*, and was not associated with the host pea plant.

A. *Experiments with Pea Plants*

The pea tissue was ground with an equal part of 0·13 M. phosphate buffer, pH 7·4, and quartz sand in a chilled pestle and mortar. The macerate was strained through four layers of muslin to yield the crude enzyme preparations.

The standard reaction mixture contained substances at the following final concentrations: αKG, 0·007 M.; γAB, 0·033 M. or DL-alanine, 0·066 M.; pyridoxal phosphate, 3×10^{-5} M.; phosphate buffer, 0·065 M.; and enzyme preparation, 2 ml. The total volume of the mixture was 3·1 ml. Appropriate control mixtures were used. Reactions were allowed to proceed for 3 hours at 35°, and were stopped by plunging the reaction tubes into a water-bath at

100° for 5 minutes. Denatured protein was removed by centrifugation, and a portion of the supernatant was used for the assay of GA, performed manometrically, using glutamic decarboxylase present in an acetone powder of *Escherichia coli*.

Transaminase activities were measured for a number of tissues from pea plants (see Table 1). In Expt. 1, nodulated peas (produced by inoculation with *Rhizobium*) and *Rhizobium*-free peas, grown under the same conditions, were used. In Expt. 2, nodulated peas were grown and divided at the hypocotyl to give root and shoot portions. In Expt. 3, the nodules were removed from the roots of approximately half a batch of nodulated peas and a com-

TABLE 1

Transamination Reactions Catalysed by Preparations from Pea Tissue

(Results expressed as μg. glutamic acid formed in 3 hours per gramme fresh weight of tissue)

Experiment	Tissues	Substrates added: αKG+γAB	Substrates added: αKG+alanine	GA from γAB / GA from alanine
1	nodule-free roots	0	900	0
	nodulated roots	260	1,000	0·26
2	nodulated plants: shoots	0	500	0
	nodulated plants: roots	290	790	0·38
3	roots+nodules	550	880	0·63
	roots−nodules	140	820	0·17
4	nodules alone	3,180	4,160	0·76

Reaction mixtures contained substances at the following final concentrations: αKG, 0·007 M.; γAB, 0·033 M. or DL-alanine, 0·066 M.; pyridoxal phosphate, 3×10^{-5} M.; phosphate buffer, pH 7·4, 0·065 M.; and enzyme preparation 2·0 ml. Total volume 3·1 ml.

parison was made with intact nodulated roots. Complete removal of the nodules was impossible since it would have entailed too much damage to the roots. In Expt. 4, nodules which had been removed were used. Due to the small amount of material available for this experiment, the total volume of the reaction mixture was reduced to 0·6 ml. but the concentration of substrates was unchanged.

No γAB-αKG transaminase activity was found in plants without nodules (Expt. 1). The striking reduction in γAB-αKG transaminase activity observed when most of the nodules were removed from roots compared with the slight reduction in the alanine-αKG transaminase (Expt. 3) indicated that the γAB transaminase activity was confined largely, if not entirely, to the nodules.

Nodules alone were shown to possess very active transaminases (Expt. 4) and the ratio between the amounts of γAB and alanine undergoing transamination attained its highest value for this tissue. It was not possible to determine whether all the γAB transaminase activity of nodules was in fact associated only with the *Rhizobium* symbiont, because the bacterium loses its cell walls during symbiotic growth; the homogenate obtained consists of an unresolved mixture of host and microbial-symbiont tissue. *Rhizobium*, grown

in pure culture, was examined, however, and found to have high γAB-αKG transaminase activity.

B. *Experiments with Other Plants*

When the techniques used to demonstrate transaminase activity in nodulated pea roots were applied to other plant materials (potato tubers, carrot roots, tulip and daffodil leaves, and mung bean seedlings), transaminase activity for γAB was not observed. Negative results were still obtained after the protein present in the plant homogenates was concentrated by ammonium sulphate precipitation or by freeze drying. Alanine-αKG transaminase, assayed as a control system, showed good activity in all preparations.

C. *Experiments with Mitochondria from Peanut Seedlings*

Certain experiments with mitochondria from peanut seedlings (see Section 2) indicated that they contained a γAB-αKG transaminase. Mitochondria were isolated from the cotyledons of 5-day-old peanut seedlings and the presence of the transaminase finally demonstrated by the following method. The mitochondria, isolated by fractional centrifugation, were suspended in 0·1 M. phosphate buffer, pH 7·4, containing 0·1 per cent. O.P.C. 45, a non-ionic detergent supplied by Petrochemicals Ltd., which led to their disruption. Washed Dowex 1 anion-exchange resin in the formate form (0·5 g.) was added to each 5 ml. of suspension, and the mixture was centrifuged at 20,000 *g.* for 10 minutes; the clear supernatant was used as the enzyme preparation. The resin removed cofactors required by glutamic dehydrogenase and so prevented the removal of any GA formed during the subsequent transamination; it also removed endogenous GA present in the mitochondrial extract, thereby making the assay of GA formed during transamination more sensitive. The final concentrations of reactants used were those previously described; 0·5 ml. of enzyme preparation was used and the total volume was 2·0 ml.

TABLE 2

Transamination of γAB *Catalysed by an Extract Prepared from Peanut Mitochondria*

	Substrate added					
	None	αKG	Pyruvate	γAB	γAB+ αKG	γAB+ Pyruvate
μg. amino-acid formed	0	0	0	0	45	130
μg. N transferred	0	0	0	0	4·3	20·5

Reaction mixtures contained substances at the following final concentrations: αKG or pyruvate, 0·007M.; γAB, 0·033 M.; pyridoxal phosphate, 3×10^{-5} M.; phosphate buffer, pH 7·4, 0·066 M.; mitochondrial extract 0·5 ml. Total volume 2·0 ml.

Pyruvate and oxaloacetate were also tested as amino-group acceptors. The results of an experiment to compare αKG and pyruvate as acceptors are given in Table 2. The amino group of γAB was transferred to pyruvate approximately five times faster than to αKG. Aspartate was not formed when oxaloacetate and γAB were used, although some alanine was produced. The latter

amino-acid would be produced by the γAB-pyruvate transaminase using pyruvate derived from the decarboxylation of oxaloacetate. These results indirectly demonstrate that no alanine-oxaloacetate transaminase was present in mitochondria.

The reversal of the transamination reaction was studied using the same concentrations of keto- and amino-acids given above. Aspartic acid, GA, and alanine were used as amino-group donors. γAB was not produced from aspartate and succinic semialdehyde (SSA). When alanine and GA were used, approximately equal amounts of γAB (410 μg. and 380 μg. respectively) were formed from SSA.

In experiments to study the intracellular distribution of the transaminase, the activity of the γAB-pyruvate transaminase was normally assayed because the activity of the γAB-αKG transaminase was too low. In one experiment, 1·07 per cent. of the total nitrogen present in the homogenate was isolated in the mitochondrial fraction and 0·3 per cent. in the microsomal fraction. The specific activity of γAB-pyruvate transaminase was highest in the mitochondrial extracts (356 μg. alanine per mg. N per hour). In the homogenate, 13 μg. alanine per mg. N per hour were formed. Microsomal extracts produced alanine, as shown by paper chromatography, but the amount was too small to estimate. Of the total transaminase activity present in an homogenate, 31 per cent. was present in the mitochondria. The remaining activity was present largely in the non-particulate, soluble fraction of the cell.

2. Oxygen Uptake Associated with γAB Metabolism

When γAB was added to brain-tissue slices (Tsukada, Nagata, and Takagaki, 1957), whole yeast cells (see preceding paper, Part 1), and cell-free extracts of *Achromobacter* (Higashi, Horio, and Okunuki, 1957), an increase in the uptake of oxygen by the preparations was observed. The oxygen uptake was enhanced further by the addition of αKG when GA was formed (Higashi, Horio, and Okunuki, 1957). These observations support the theory that a transamination reaction forms the primary step in γAB degradation and that the oxygen uptake can be attributed to the oxidation of the products of transamination, namely SSA and GA. Tsunoda and Shiio (1959) showed that whole cells of *Bacillus pumulis* still metabolized γAB with associated oxygen uptake even in the presence of arsenite and they assumed that a direct oxidation of γAB was occurring. However, another explanation for such arsenite-resistant oxygen uptake was given in Part 1; in addition, Nirenberg and Jakoby (1960) have shown that SSA oxidation may not be completely inhibited by arsenite.

Direct β-oxidation of γAB has been demonstrated in brain tissue leading to the formation of γ-amino-β-hydroxybutyric acid (Seo, 1957; Inui, 1959).

Experiments with Mitochondria from Peanuts

Particulate preparations from cotyledons of peanut seedlings were used in the experiments to test whether β-oxidation of γAB occurs in plants. Peanut

cotyledons were chosen because they form a good source of the enzymes involved in β-oxidation of fatty acids.

Mitochondria were isolated from the cotyledons by fractional centrifugation and then they were suspended in a medium containing 0·25 M. sucrose, 0·05 M. phosphate buffer, pH 7·4, $2{\cdot}5\times10^{-5}$M. sodium ethylenediaminetetra-acetate. This suspension (0·5 ml.) was added directly to the substrates and

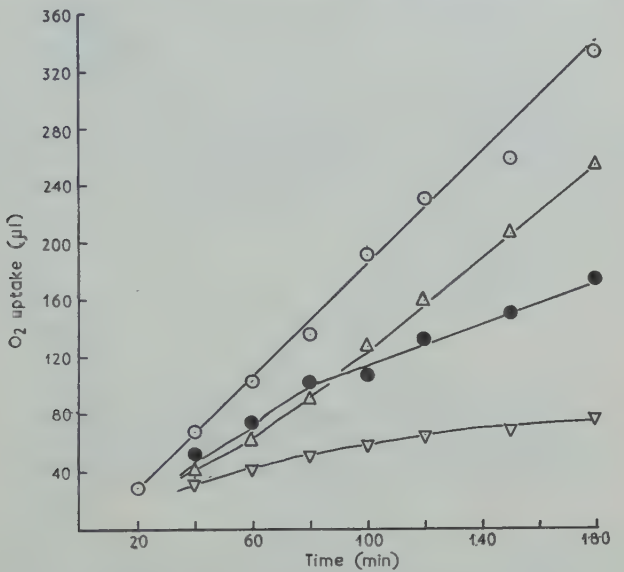

FIG. 1. Extra oxygen uptake produced by peanut mitochondria when γAB was added to different concentrations of αKG.

αKG 0·001 M. (▽—▽), αKG 0·001 M.+γAB (●—●).
αKG 0·01 M. (△—△), αKG 0·01 M.+γAB (○—○).

Readings corrected for endogenous O_2 uptake. System contained substances at the following final concentrations: αKG, 0·01 or 0·001 M.; γAB, 0·01 M.; cysteine, 5×10^{-4} M.; ATP, 1×10^{-3} M.; DPN, $7{\cdot}4\times10^{-5}$ M.; TPN, $6{\cdot}6\times10^{-5}$ M.; $MgSO_4$, 0·01 M.; sucrose, 0·25 M.; phosphate buffer, pH 7·4, 0·05 M.; EDTA, $2{\cdot}5\times10^{-3}$ M. Total volume 2·0 ml.

cofactors which were present in the reaction mixtures (total volume, 2 ml.) at the following final concentrations: γAB, 0·01 M.; cysteine, 5×10^{-4} M.; Mg^{++}, 0·01 M.; ATP, 0·001 M.; DPN, $7{\cdot}5\times10^{-5}$ M.; TPN, $6{\cdot}6\times10^{-5}$ M.; sucrose, 0·25 M.; phosphate buffer (pH 7·4), 0·05 M. When tricarboxylic acid cycle intermediates were added, final concentrations of 0·001 M. acid were used.

When γAB was the sole acid added to the mitochondria, no increase in their endogenous rate of oxygen uptake occurred. If αKG was added together with γAB, an amount of oxygen, extra to that observed with αKG alone, was taken up (compare the results with *Torulopsis*, Part 1). The rate of this extra oxygen uptake was largely independent of the concentration of αKG used (see Fig. 1).

A similar result was obtained when mixtures of succinate and γAB were used, and the rate of extra oxygen uptake observed for this mixture was the same, after an initial short lag period, as that observed with the mixture of γAB and αKG (see Fig. 2). Other tricarboxylic acid cycle intermediates when supplied with γAB also gave rise to such extra oxygen uptakes. The rates of

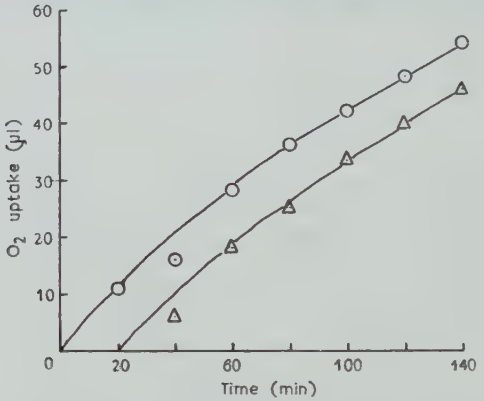

FIG. 2. Rates of extra oxygen uptake by peanut mitochondria on addition of γAB to αKG and succinate. αKG and succinate 0·001 M., and other conditions as in Fig. 1. Extra rate with αKG (○—○), extra rate with succinate (Δ—Δ).

extra oxygen uptake (stated in parentheses as μl. O_2 per hour) were closely similar for all acids, e.g. succinate (21·5), fumarate (22·0), citrate (18·5), and pyruvate (18·7). This similarity suggests that a common rate-controlling reaction governs oxygen uptake during γAB degradation in the presence of any other organic acid. The oxidation of SSA, formed from γAB and αKG or pyruvate by transamination, suggests itself as the probable rate-controlling reaction, although the two possible transamination reactions show only low activity (see section 1, C). This explanation involves the well-established fact that mitochondria can convert the various acid intermediates into pyruvate or αKG rapidly (Millerd *et al.*, 1951).

The nitrogen-containing products of the reactions occurring when γAB was supplied to mitochondria together with αKG or succinate were examined by paper-chromatographic methods. Aspartic acid and alanine were identified, but no GA could be detected. The quantities of nitrogen (originally present in γAB) found in other compounds after metabolism varied with the additional carboxylic acid used (Table 3). Amino-acids were estimated by quantitative paper-chromatographic methods and ammonia by a nesslerization procedure.

Arsenite (0·001 M.) completely inhibited the oxidation of αKG by mitochondria for at least 50 minutes. When arsenite was added to the reaction mixture containing γAB and αKG, oxygen uptake was observed after 20 minutes (see Fig. 3). Aspartate and alanine were again the products of the reaction.

TABLE 3

Distribution of Nitrogen from γAB after Metabolism

(Values are increases of μg. N present in each compound per μmole of oxygen uptake)

	Substrates added	
	γAB+αKG	γAB+succinate
Aspartate	4·7	5·2
Alanine	2·1	4·8
Ammonia	7·0	2·6
Total	13·7	12·6

Reaction mixtures contained substances at the following final concentrations: γAB, 0·01 M.; αKG or succinate, 0·001 M.; cysteine, 5×10^{-4} M.; ATP, 0·001 M.; DPN, $7{\cdot}5 \times 10^{-5}$ M.; TPN, $6{\cdot}6 \times 10^{-5}$ M.; $MgSO_4$, 0·01 M.; sucrose, 0·25 M.; phosphate buffer, pH 7·4, 0·05 M. (and EDTA, $2{\cdot}5 \times 10^{-3}$ M.). Total volume 2·0 ml.

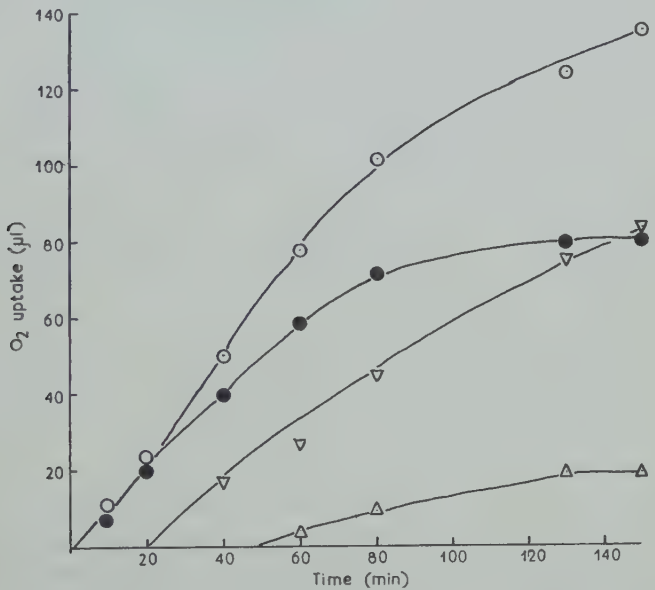

FIG. 3. Effect of arsenite upon O_2 uptake of peanut mitochondria in the presence of αKG and γAB. Arsenite, 0·001 M.; αKG, 0·001 M.; and other conditions as in Fig. 1.
αKG (●—●), αKG and γAB (○—○), αKG and arsenite (△—△), αKG, γAB, and arsenite (▽—▽).

Presumably these amino-acids were produced by transamination reactions. Alanine would be formed in a reaction between γAB and pyruvate arising from succinate or, more slowly, from αKG. γAB-oxaloacetate and alanine-oxaloacetate transaminase activities could not be detected in the mitochondria (see section 1, C), and therefore the formation of aspartate was probably dependent upon an active GA-oxaloacetate transaminase. The absence of GA from the reaction mixtures is attributed to the action of coupled enzyme systems. Normally, GA would accumulate as a product of *in vitro* transamination between γAB and αKG, a reaction which is seen as the logical

initial step leading to γAB degradation. However, in the intact, strictly-organized mitochondria, the distribution of substrates and enzymes may be such that the GA formed was utilized immediately either by glutamic dehydrogenase or by GA-oxaloacetate transaminase yielding ammonia and aspartate respectively. These reaction sequences would be favoured by a close juxtaposition of the transaminases and malic dehydrogenase (producing oxaloacetate) within the mitochondria.

These experiments provided no evidence to support the operation in plants of a β-oxidation reaction leading to the formation of γ-amino-β-hydroxybutyric acid initially, or glycine by a further oxidation.

3. Metabolism of C^{14}-γAB

When generally labelled C^{14}-γAB was injected into intact rats and mice, the C^{14}-label was incorporated into succinate, aspartate, glutamate, and alanine (Roberts, Rothstein, and Baxter, 1958; Wilson, Hill, and Koeppe, 1959). This distribution indicated that the carbon skeleton formed after the initial transamination of γAB was metabolized by the reactions of the tricarboxylic acid cycle. Additional support for this view was provided by Tsukada, Nagata, and Takagaki (1957) who showed that $C^{14}O_2$ was evolved rapidly when [1-C^{14}]γAB was supplied to brain slices. The reactions of the tricarboxylic acid cycle also appear to be responsible for the formation of glutamate from γAB in *Bacillus pumulis* (Tsunoda and Shiio, 1959).

Steward, Bidwell, and Yemm (1958) supplied C^{14}-γAB to tissue cultures of carrot root and found that a large proportion of the activity appeared in glutamine. In contrast, when C^{14}-glutamine was supplied to this tissue, little activity appeared in γAB. Glutamine was formed from γAB 70 times faster than γAB was produced from glutamine. These data could support the idea that γAB was carboxylated to form glutamate, which was converted subsequently into glutamine. However, their usefulness in indicating the metabolic pathway traversed by γAB was very limited because the shortest time interval used to study its degradation was 22 hours. In this time, the C^{14}-label could have passed through many transient intermediates before its final accumulation in glutamine.

The results now reported show that in leaf discs of peas and in mitochondria from cotyledons of peanuts the C^{14}-label of C^{14}-γAB quickly entered the tricarboxylic acid cycle intermediates and their related amino-acids (aspartate, alanine, and glutamate) and that, in the case of pea leaves, radioactivity was present predominantly in the amide fraction after 24 hours.

A. *Experiments with Leaf Discs of Peas*

The leaf discs were allowed to metabolize C^{14}-γAB for various periods of time up to 24 hours. The discs (4·5 mm. diameter) were cut from pea leaves, the midrib being excluded. For each metabolic period, a solution of C^{14}-γAB was introduced into eight discs (total fresh weight about 0·1 g.) by vacuum infiltration. In this way, each batch of leaf discs received about 0·05 μc.

(50,000 c.p.m.) of C^{14}-γAB. The discs were washed several times with distilled water and then placed in 0·5 ml. of distilled water in Warburg flasks containing a 15 per cent. KOH solution in the centre wells. The flasks were shaken at 25° in the dark. The leaf discs were killed at appropriate times by treating with boiling ethanol. Vacuum infiltration and subsequent washing of the leaf discs took about 3 minutes. Therefore in the zero time sample, it was possible that some metabolism occurred before the leaf tissue was killed. In one experiment, other samples of leaf discs were killed after 10, 30, and 60 minutes, whilst in a second experiment samples were taken at 1, 2, 4, 6, and 24 hours.

Respiratory $C^{14}O_2$ trapped in the centre wells of the Warburg flasks was converted into barium carbonate and its radioactivity was determined using a methane gas-flow proportional counter.

The distribution of radioactivity among compounds soluble in 75 per cent. ethanol was investigated by radioautographic techniques. After concentration of the ethanolic extracts, measured portions were spotted on to sheets of Whatman 3 MM filter-paper, and two-dimensional chromatograms were prepared using phenol-ammonia followed by an *n*-butanol–acetic acid–water mixture as the solvent systems. Radioautographs were prepared from the chromatograms using Kodirex X-ray film. It was not possible to determine specific activities upon the small amounts of material separated upon the chromatograms.

TABLE 4

Amount of Activity (counts per minute) Respired as $C^{14}O_2$ *from Pea-leaf Discs supplied with* C^{14}-γAB (*about* 5×10^4 *c.p.m./0·1 gm. fresh weight*)

	Hours after supplying C^{14}-γAB to leaf discs				
	1	2	4	6	24
CO_2 respired	660	2,350	3,250	11,500	46,250

The radioactivity present in respiratory CO_2 was too low to determine in samples of leaf material killed after periods of less than 1 hour. Values determined for the longer time periods are given in Table 4. By extrapolation, half of the C^{14} initially supplied as γAB appeared in the respiratory CO_2 in about 10 hours. If the C^{14}-γAB equilibrated rapidly with unlabelled acid normally present in the leaf, then the half-life of a γAB molecule must be less than 10 hours, since many reactions must occur before CO_2 is produced from all the carbon atoms.

The sequence in which the ethanol-soluble compounds became labelled can be seen in Fig. 4. In the brief period necessary for infiltration and washing (3 minutes), radioactivity appeared in aspartic acid (radioautograph not reproduced in Fig. 4). After 10 minutes (Fig. 4*a*), aspartic acid, alanine, and GA contained labelled carbon. Radioactivity was first detected in asparagine and glutamine at 30 minutes (Fig. 4*b*). Subsequently the amides progressively became more heavily labelled, and at 6 hours (Fig. 4*c*) they contained more C^{14} than did GA and aspartic acid. Succinate, other organic acids, and sugar

phosphates were also labelled in the 6-hour leaf sample. Except for residual γAB, more activity was present in asparagine than in any other compound after 24 hours (Fig. 4*d*).

This sequence of amino-acid labelling is that which would be expected if γAB was metabolized by reactions of the tricarboxylic acid cycle; the labelling of succinate also confirms that the carbon skeleton of γAB enters intermediary metabolic processes.

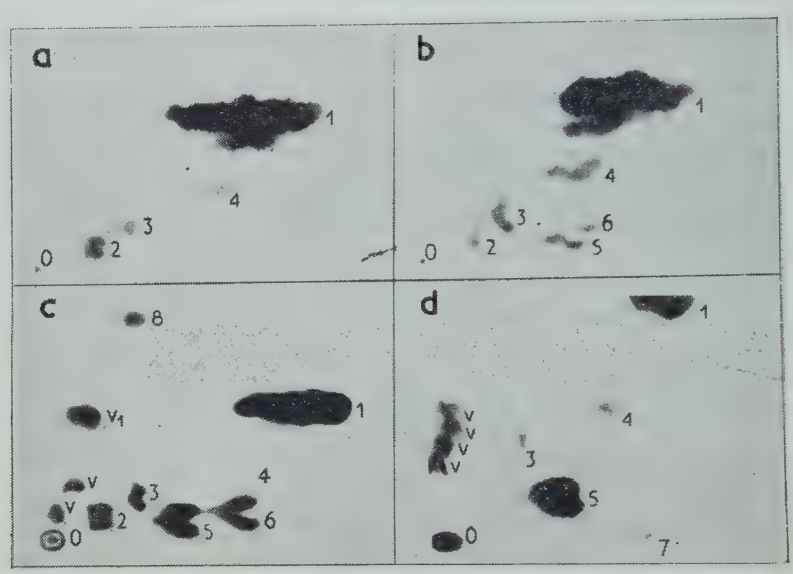

FIG. 4. Reproduction of radioautographs prepared from extracts of pea-leaf discs infiltrated with C^{14}-γAB. The leaf discs were extracted (*a*) 10 minutes, (*b*) 1 hour, (*c*) 6 hours, (*d*) 24 hours after infiltration. The extracts were applied at the bottom left hand corner of the chromatograms, which were developed in phenol-NH_3 (flow along horizontal direction) followed by *n*-butanol–acetic acid–water mixture (vertical). The radioactive spots were identified as 1, γAB; 2, aspartic acid; 3, glutamic acid; 4, alanine; 5, asparagine; 6, glutamine; 7, unidentified compound; 8, succinic acid; V, unknown sugar phosphates, and V_1, unknown acid. O, original position of spot.

B. *Experiments with Mitochondria from Peanut Cotyledons*

An homogenate of the cotyledons of peanut seedlings was centrifuged to give mitochondrial, microsomal, and residual, soluble (supernatant) fractions, each of which was used as an enzyme source. The concentrations of substrates and cofactors supplied to these preparations were the same as those used in oxidative experiments (section 2 above), apart from C^{14}-γAB (total amount 1·03 μg., 0·1 μc.) whose concentration was negligible. The reaction mixtures (total volume 0·1 ml.) were placed in the side arms of Warburg flasks which were shaken at 30°. Respiratory CO_2 was trapped and its radioactivity determined as above.

The ethanol-soluble substances of each reaction mixture were separated into amino-acid and organic-acid fractions by adsorption upon and elution

from ion-exchange resin columns of Zeocarb 215 and Dowex 1 respectively. The distribution of radioactivity within the amino-acid fractions was determined by radioautographic techniques as above. In a similar way, two-dimensional chromatograms were prepared from portions of the organic-acid fractions; aqueous phenol was used as the first solvent and the lower phase from a mixture of chloroform, *t*-amyl alcohol, formic acid and water (8:8:3:8 parts by volume as Bentley, 1952) provided the second solvent. Radioautographs were then prepared as above.

Apparently γAB was not metabolized by the microsomal and supernatant fractions; no radioactivity was present in the respiratory CO_2 and no radioactive spots were detected on chromatograms other than that due to unchanged C^{14}-γAB.

TABLE 5

Amount of Activity (counts per minute) Present in CO_2 *Evolved from Mitochondria Supplied with* C^{14}-γAB (*about* 10^5 *c.p.m. per 0·1 ml. reaction mixture*)

		Hours incubated				
	Substrate	0·5	1·0	1·5	2·0	3·0
Experiment 1						
	C^{14}-γAB	—	5,450	—	15,500	24,600
	C^{14}-γAB+succinate	—	1,500	—	10,000	21,000
Experiment 2						
	C^{14}-γAB	—	—	3,700	—	11,700
	C^{14}-γAB+succinate	—	—	3,100	—	7,700
	C^{14}-γAB+αKG	—	—	3,200	—	9,700

Reaction mixtures contained substances at the following final concentrations: αKG or succinate, 0·001 M.; DPN,7 $\cdot 5 \times 10^{-5}$ M.; TPN, $6 \cdot 6 \times 10^{-5}$ M.; cysteine, 5×10^{-4} M.; ATP, 0·001 M.; $MgSO_4$, 0·01 M.; sucrose, 0·25 M.; phosphate buffer, pH 7·4, 0·05 M., and EDTA, $2 \cdot 5 \times 10^{-3}$ M. Total volume 0·1 ml.

When mitochondria were used the sequence in which amino-acids became labelled was similar to that observed with leaf discs of pea except that no activity entered the amides. This finding was not unexpected because Loomis (1959) showed that glutamine synthetase activity was confined to the non-particulate cytoplasmic fraction of peanut seedlings.

In contrast to results obtained in the larger-scale oxidative experiments using unlabelled γAB (section 2 above), C^{14}-γAB could be metabolized by mitochondria without the addition of a tricarboxylic acid cycle intermediate. Presumably the endogenous keto-acids present in the mitochondria were sufficient for transamination of the small amounts of labelled γAB used. Respiratory CO_2 evolved in the first few hours contained smaller amounts of radioactivity when either succinate or αKG were added together with C^{14}-γAB (see Table 5, Expts. 1 and 2).

The same compounds were labelled in the amino-acid fractions from mitochondria supplied with C^{14}-γAB either alone or together with succinate, but

C^{14}-incorporation into amino-acids of mitochondria receiving succinate was less during the first hour of metabolism. In contrast, a striking difference was noted in the radioactivity present in the organic-acid fractions from mitochondria subjected to these two treatments. When C^{14}-γAB was supplied together with succinic acid, five acids became labelled. Malic and fumaric acids contained more total radioactivity than succinic, citric, and αKG acids (see Fig. 5); specific activities could not be determined on the trace amounts of the acids involved. Negligible radioactivity was present in the organic-acid fraction from the mitochondria that received only C^{14}-γAB.

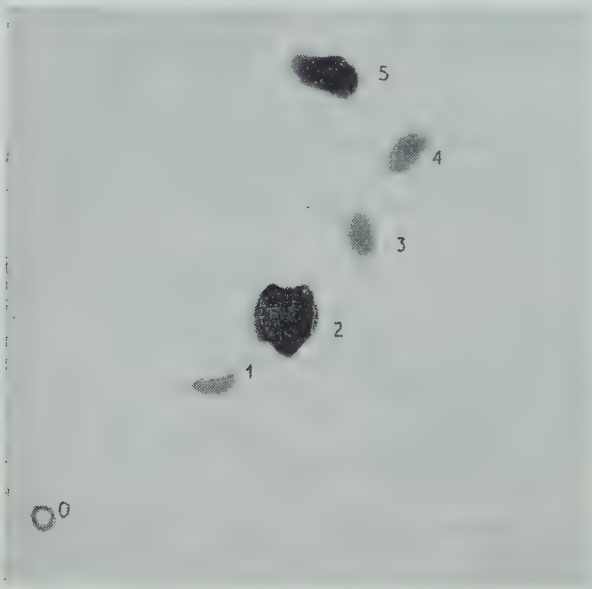

FIG. 5. Reproduction of radioautograph prepared from the organic-acid fraction of peanut mitochondria which were supplied with C^{14}-γAB and incubated for 1 hour. The spot was applied to the bottom left-hand corner of the chromatogram which was developed in aqueous phenol (flow in horizontal direction) and chloroform–*t*-amyl alcohol–formic acid–water mixture (vertical). The radioactive spots were identified as: 1, citrate; 2, malate; 3, αKG; 4, succinate; and 5, fumarate. O, original position of spot.

These observations can be explained by assuming that the concentrations of the tricarboxylic acid cycle intermediates present in the isolated mitochondria were low and that the turnover rates of individual acid molecules were high. Therefore, when C^{14}-γAB was supplied alone, C^{14}-atoms would pass through the cycle intermediates rapidly, being quickly withdrawn as C^{14}-amino-acids or evolved as $C^{14}O_2$. When exogenous succinate or αKG (which would yield succinate by oxidative decarboxylation) were available to mitochondria, the sizes of the metabolic pools of these and other tricarboxylic acid cycle intermediates present within the mitochondria would be increased.

C^{14}-labelled molecules of succinate, arising from C^{14}-γAB supplied simultaneously, would be diluted by a large number of unlabelled molecules. The active molecules then would participate in Krebs cycle reactions at a reduced rate and so radioactivity would tend to accumulate in the organic-acid fraction, whilst the rate of C^{14}-incorporation into amino-acids and respiratory CO_2 would be reduced.

These experiments with pea leaves and mitochondria from peanuts therefore support the view gained using animal and microbial systems that GA was formed from γAB only after the carbon skeleton of the latter acid had passed through intermediates of the tricarboxylic acid-cycle. No evidence was obtained in support of the view that higher plant tissues can carboxylate γAB directly to give GA.

4. The Formation of γAB

γAB is formed by decarboxylation of GA. The enzyme concerned, glutamic decarboxylase, has been studied in extracts of many plants, whilst its properties have been examined most critically in extracts of barley roots (Beevers, 1951). However, little information is available concerning the intracellular distribution of this enzyme.

Rogers (1955) subjected an homogenate of acorn squash to fractional centrifugation and concluded that GA decarboxylase was mainly present in the non-particulate cytoplasmic fraction remaining after mitochondria and microsomes had been removed. However, the mitochondria and microsomes were unlikely to remain intact during the homogenization procedure since no attempt was made to maintain osmotic equilibrium. Therefore the results obtained cannot be accepted as a valid demonstration that GA decarboxylase is not a particle-bound enzyme.

In the present experiments, peanut cotyledons were shown to contain only low GA decarboxylase activity. Mung bean seedlings were a more suitable plant material in which to study the intracellular distribution of the enzyme, whose activity was relatively high in two-day-old seedlings. Considerable quantities of the enzyme were known earlier to be present in the dry seed (Kulkarni and Sohonie, 1956).

Mitochondria, microsomes, and soluble supernatant fractions were obtained from seedlings as described earlier, but, in this case, the phosphate buffer present in the homogenizing medium was pH 6·0. The mitochondria and microsomes were suspended finally in 0·1 M. phosphate, pH 5·2, containing 0·1 per cent. O.P.C. 45, and GA and phosphate buffer, pH 5·2, were added to give reaction mixtures containing final concentrations of 0·01 M. and 0·3 M. respectively. Evolution of CO_2 was followed manometrically at 30° over a 3-hour period. Controls without added GA were used.

No GA decarboxylase activity was found in the microsome fraction. The rate of CO_2 production (1,400 μl. per hour per mg. N present in the fraction) obtained with the supernatant fraction was constant over the first 20 minutes, and CO_2 evolution continued until the experiment was terminated, when

about 25 per cent. of the GA supplied initially had been decarboxylated. Only slight CO_2 production was observed with the mitochondrial fraction and so it was possible only to obtain a mean value of the rate (66 μl. per hour per mg. N) for the whole 3 hours. This value may be lower than the true initial rate. The rate obtained using the whole homogenate did not differ significantly from that obtained with the supernatant fraction.

Glutamic decarboxylase is then largely present in the soluble supernatant fraction. The small activity associated with the mitochondrial fraction may result from a slight adsorption of enzyme upon the surface of the particles from the supernatant phase.

General Discussion

A more complete understanding of the metabolic fate of the γAB molecule in higher plants has been built up by the experiments reported, and an attempt has been made to examine the sub-cellular distribution of the enzymes immediately concerned in its biosynthesis and biodegradation. Apparently the enzymic reaction yielding γAB proceeded almost exclusively in the non-particulate cytoplasm, whilst the transaminase responsible for the initial degradative reaction was concentrated particularly in mitochondria, although a considerable proportion of the total enzyme activity resided in the supernatant fraction. Transaminases involved in the metabolism of other amino-acids also have been reported to be present in mitochondria, e.g. in lupin seedlings (Wilson, King, and Burris, 1954) and in mung bean seedlings (Bone and Fowden, 1960).

Conclusions concerning sub-cellular organization are subject to the uncertainties inherent in the use of the differential centrifugation technique for the separation of the sub-cellular fractions. For instance, it is impossible to establish that the soluble supernatant fraction has not been contaminated by substrates and enzymes released by particles damaged during maceration or subsequent centrifugation. Alternatively, some adsorption of soluble components upon mitochondrial or microsomal surfaces during isolation appears feasible. Doubt also surrounds the nature of the particulate fractions themselves, for although they are referred to as mitochondria and microsomes following general usage, the terms do no more than describe material possessing certain sedimentation coefficients, and a number of recent reports indicate that considerable heterogeneity exists within each fraction.

In the past it has been established that the γAB contents of different plant tissues cannot be related directly to their contents of GA decarboxylase (see Introduction, Part 1). Now it would seem that the amount of γAB in particular tissues may be controlled in part by the rates of intracellular movement of two types of molecule. First GA must be released from the mitochondria where it is synthesized by the action of glutamic dehydrogenase (Bone, 1959), into the non-particulate cytoplasm, where GA decarboxylase exists, before γAB is produced. Then, before γAB degradation can proceed, many of the latter molecules must move back to the mitochondria, where the highest con-

centrations of transaminase occur. The balance existing between these two transport processes will tend to regulate γAB turnover and may be very different in different plant tissues.

Most of the reactions of the by-pass metabolic pathway set out in the Introduction to Part 1 have been shown to occur in higher plants. Glutamic dehydrogenase and GA decarboxylase actions can be demonstrated readily in extracts of many plants, whilst the existence of a γAB transaminase is now definitely established. As yet, SSA dehydrogenase activity has not been shown to be present in higher plants, but the radioisotopic experiments described in section 3 leave no doubt that the carbon skeleton of the γAB molecule is converted into succinate. The minimum requirements necessary for the operation of a by-pass metabolic pathway then appear to be satisfied by higher plants, but, as in the case of yeasts, it is difficult to predict what proportion of αKG molecules leave the tricarboxylic acid cycle and enter this alternative pathway. The fact that the enzymes involved in the by-pass reactions are present in different sub-cellular fractions suggests that the efficiency of the whole pathway may be rather low.

As suggested in Part 1, the by-pass reactions may assume a greater importance under conditions which restrict the normal conversion of αKG into succinic acid. γ-Methyl-γ-hydroxy-α-ketoglutaric acid, or more strictly its lactone, which is produced by condensation (dimerization) of pyruvic acid, acts as an inhibitor of the oxidative decarboxylation of αKG (Montgomery and Webb, 1954). It is interesting to note that a related keto-acid, γ-methylene-α-ketoglutaric acid, is a constituent of a number of plants including *Lilium regale* (Wickson and Towers, 1956), tulip (Towers and Steward, 1954), and peanuts (Fowden and Webb, 1955); the corresponding amino-acid, γ-methyleneglutamic acid, is present in the plants at far higher concentrations. The structural similarity of the two keto-acids is sufficiently close to suggest that the naturally occurring γ-methylene acids also may inhibit the oxidative decarboxylation of αKG and so, in the above three species, the reactions of the by-pass mechanism may have enhanced importance.

Acknowledgements

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Changes in the Level of the Protein Nitrogen during Growth of the Gametophyte and the Initiation of the Sporophyte of *Dryopteris borreri* Newm.

BY

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Abstract

Estimations of the amounts of organic nitrogen in spores and developing gametophytes of *Dryopteris borreri*, a fern reproducing apogamously and lacking archegonia, have shown that the nitrogen in the gametophytes increases exponentially. The logarithmic rate of increase of the nitrogen remains unchanged during the initiation and emergence of the sporophyte. Gametophytes about to produce sporophytes yielded only filamentous growths when transferred to a medium containing 8-azaguanine, and the increase in their content of organic nitrogen was very small. The results are discussed in relation to similar estimations made by other workers upon the gametophytes of *Dryopteris erythrosora* (Eat.) Schott, a fern reproducing sexually. The interpretation placed upon the results obtained for *D. erythrosora* is questioned, and a new interpretation of these results, together with those for *D. borreri*, is related to the difference in morphology of the two generations in the life cycle of the Polypodiaceous ferns.

Introduction

THE experiments of Hotta and Osawa (1958) have shown that accompanying the increasing morphological complexity of the gametophyte of *Dryopteris erythrosora*, a sexually reproducing fern, there is a rise in the proportion of protein nitrogen in the gametophyte, particularly marked at the time of the change-over from the filamentous to the cordate form. The experiments described here were designed to see, firstly, whether in *Dryopteris borreri*, a fern reproducing apogamously but in which the ontogeny of the gametophyte is similar to that in *D. erythrosora*, corresponding changes occurred in the protein nitrogen. Secondly, since in *D. borreri* the sporophyte appears directly from the gametophyte without any pause in growth, the experiments were continued during the initiation and emergence of the sporophyte. The aim was to discover whether this second and more striking morphogenetic change was accompanied by another sharp rise in the proportion of the protein in the growing system. Two rises in the proportion of protein were in fact discovered in *D. borreri* accompanying the two morphological gradations between the spore and the sporophyte. Further consideration of these results and of those obtained for *D. erythrosora* has led, however,

[1] The experimental work described in this paper was carried out by the junior author and formed part of a thesis submitted for the M.Sc. degree of the University of London.

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to an interpretation of the metabolic changes occurring in the developing gametophyte different from that advanced by Hotta and Osawa.

Hotta and Osawa also reported that in the presence of a low concentration of 8-azaguanine cultures of *D. erythrosora* remained in a filamentous condition. This effect was different from that of the ordinary inhibitors of growth; these merely reduced the growth-rate without stopping or deflecting morphogenesis. Experiments were therefore made to see whether this purine analogue was equally effective in limiting the development of *D. borreri*, and in particular whether by supplying it to gametophytes in which the initiation of the sporophyte was imminent, morphogenesis could be prevented or deflected.

Materials and Methods

Pure cultures of the gametophytes of *Dryopteris borreri* (triploid race) were obtained from spores collected in the wild, sterilized in calcium hypochlorite solution, and sown on slopes of Moore's medium solidified with 1·5 per cent. agar. The cultures were kept at room temperature in diffuse daylight supplemented by continuous artificial illumination.

The course of the development of *Dryopteris borreri* in culture was determined from germination (occurring about a week after sowing) until the young sporophyte was readily visible to the naked eye. The form and area of the gametophytes were recorded throughout this development, and particular attention was paid to the initiation of the sporophyte, both internally as a group of densely staining cells, and externally as a minute axis arising sub-apically. A number of stages of development, which could be accurately defined and which were representative of the morphogenesis observed, were then selected for further investigation. Since a large part of each culture grew more or less uniformly, it was possible to record the age at which a culture would yield abundant material of a selected stage. The stages selected and the ages of the cultures in which they predominated are given in Table 1.

Table 1

Stages in Gametophyte Development

Age (days)	Nature of gametophyte (areas ± 10%)	Length of sporophytic axis protruding from gametophyte (± 10%)
0	Spore	None
25	Filament with no specialized apical cell	None
40	Predominantly a filament, but possessing a cuneate apical cell flanked by 2–3 cells cut from it	None
55	Cordate plate, area 2 sq. mm.	None
75	Cordate plate, area 4 sq. mm.	None
90	Cordate plate, area 6 sq. mm.	None
105	Cordate plate, area 9 sq. mm.	0.05 mm.
120	Cordate plate, area 11 sq. mm.	0·50 mm.

In a second series of cultures, which grew at a rate slightly greater than that of the first, particular attention was paid to the emergence of the sporophyte. The sporophyte arises behind the apical notch of the gametophyte (the histology of this stage has been described by Duncan (1943) and others). In the conditions of the experiments described here the organization and activity of the meristem of the gametophyte were not impaired during the early development of the sporophyte, and the gametophyte continued to expand in area. To obtain a picture of the behaviour of the two growth forms independently during the emergence of the sporophyte, the area of the gametophyte was recorded after the removal of the sporophyte with a micro-manipulator. The stages selected for further investigation are given in Table 2.

TABLE 2

Relative Sizes of Gametophyte and Sporophyte

Age (days)	Area of gametophyte with sporophyte removed (sq. mm. ± 10%)	Length of sporophytic axis (mm. ± 10%)
105	11	0·25
120	13	0·50
135	15	0·75
150	17	1·00

Cultures required for the estimation of organic nitrogen at the different stages of growth were raised in the same way as in the pilot experiments, with the exception of those providing the filamentous stages. It was found that these could not be harvested from agar without extensive damage. Consequently spores were sown on slopes of filter-paper dipping into Moore's medium. The rate of growth of these gametophytes was not appreciably different from those on agar and the filaments so obtained could be lifted from the paper with a needle without difficulty.

For the estimation of nitrogen in the spores, about 4,000 (weighing between 0·1 and 0·2 mg.) were taken from the same sample as that from which the cultures had been raised. The estimation of nitrogen in a filamentous stage was made upon the material harvested by lifting from the culture five to ten times with a needle, the total fresh weight of the gathering being of the order of 0·2 mg. For the cordate stages, six gametophytes were taken for each estimation. Each estimation was made in triplicate, and all the material analysed conformed to the stages of development defined.

In the second series of cultures, in which particular attention was paid to the amount of nitrogen present after the initiation of the sporophyte, six gametophytes were again used for each estimation. After the sporophytes had been removed from the gametophytes, the nitrogen in the two growth forms was estimated separately. These estimations were also made in triplicate.

To investigate the effect of 8-azaguanine upon the initiation of the sporophyte, preliminary experiments were made to determine that concentration of 8-azaguanine which, when incorporated into the medium, prevented the progression of the gametophyte from the filamentous to the cordate stage. Gametophytes 90 days old and 6 sq. mm. in area, the stage at which, judging from the observations made upon the pilot cultures, the initiation of the sporophyte was imminent, were then transferred to a medium containing this concentration of 8-azaguanine. At the same time controls were transferred to the basic medium alone, and other gametophytes to a medium supplemented with equal amounts of 8-azaguanine and guanine. Six gametophytes were used to determine the amount of organic nitrogen initially present, and six from each treatment at the conclusion of the experiment after 30 days' growth. This experiment was done in triplicate.

Before determining the amount of organic nitrogen present at the different stages of growth, the material was dried to a constant weight at 105° C. The weight of spores, however, fluctuated irregularly during drying, presumably because of their hygroscopic properties, and in consequence the nitrogen in the spores was expressed as a proportion of the fresh weight. The amount of moisture present in the spores appeared to be very small, and it was assumed that taking the fresh weight as equivalent to the dry weight would introduce no great error into the results. Following digestion in small Kjeldahl flasks, the nitrogen was estimated by the use of Conway units and Nessler's reagent, the intensity of the colour being measured spectrophotometrically at a wavelength of 450 mμ.

Results

Changes in the amount of nitrogen present during growth. As the mass of the gametophyte increases, so does the amount of organic nitrogen it contains. The mean amounts present at the different stages of growth of the cordate gametophyte are shown in Fig. 1A. When the amount of nitrogen is expressed as a proportion of the dry weight of the gametophyte, it is seen that after an initial fall following germination there is a continued rise, becoming sharper at the time of the change-over from the filamentous to the cordate form (Fig. 1, A). Following this transition the increase in the proportion of nitrogen becomes less marked, but subsequently there is a second and even sharper rise beginning a little before the sporophyte becomes detectable and continuing during its emergence.

The results of the more detailed examination of the early development of the sporophyte are shown in Fig. 1, B. The increasing mass of the gametophyte is again accompanied by an increasing content of organic nitrogen, but there is little change in the proportion present. There is a strikingly different situation in the embryonic sporophyte. Here, although the sporophyte underwent a fourfold increase in length and a threefold increase in dry weight in the course of the experiment, there was only a very slight progressive increase in the amount of organic nitrogen present. There is consequently a rapid fall

in the proportion of nitrogen in the sporophyte as it emerges from the gametophyte.

The effect of 8-azaguanine. The preliminary experiments showed that spores germinated normally in the presence of 8-azaguanine at a concentration of 0·085 p.p.m., but that growth remained in a filamentous condition indefinitely and cordate gametophytes failed to appear. At a concentration of

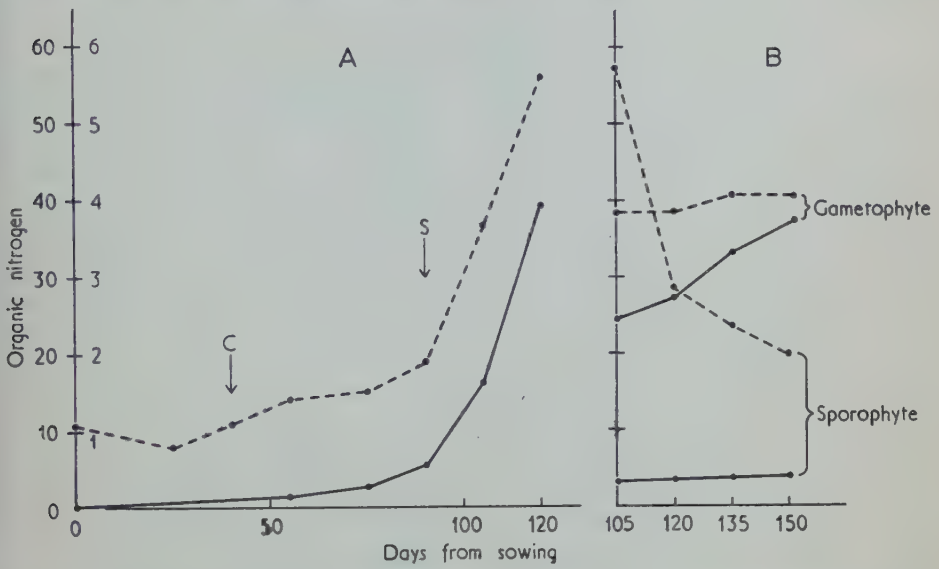

FIG. 1. The amounts and proportions of organic nitrogen in gametophytes and sporophytes of *Dryopteris borreri* at different stages of growth: ●——●, organic nitrogen as 10 μgs.; •- - -•, proportion of nitrogen as μg./mg. of dry weight. A, without separation of gametophyte and sporophyte in the later stages of growth: C, appearance of cordate form; S, initiation of sporophyte imminent. B, organic nitrogen estimated in gametophyte and sporophyte separately.

8-azaguanine below 0·055 p.p.m. growth and development were unaffected, whereas above 0·109 p.p.m. the spores failed to germinate at all. Consequently a concentration of 0·085 p.p.m. was chosen as being suitable for further experiments upon the cordate gametophytes.

When gametophytes in which the initiation of the sporophyte was imminent were transferred to the basic medium supplemented with 0·085 p.p.m. of 8-azaguanine, expansion of the gametophyte stopped immediately. After a few days single filaments were observed arising from the margin. Control gametophytes which had been transferred at the same time to unsupplemented slopes, and those transferred to slopes supplemented with both guanine and 8-azaguanine, continued to grow normally. After 30 days the area of these gametophytes had increased to about 10 sq. mm. and at the same time the sporophytic axis had appeared and reached 0·5 mm. in length. No sporophytes had been produced from the gametophytes transferred to the medium

TABLE 3

Dryopteris borreri. The Amounts and Proportion of Organic Nitrogen in Gametophytes supplied with 8-Azaguanine and Guanine

Material sampled at beginning of experiment			Amounts and proportion of nitrogen after 30 days' culture								
			Control			Medium supplemented with 0·085 p.p.m. 8-azaguanine			Medium supplemented with 0·085 p.p.m. 8-azaguanine and guanine		
Mean dry wt. of single gametophyte (mg.)	Amount of nitrogen (μg.)	Proportion of nitrogen (μg./mg. dry wt.)	Mean dry wt. of single gametophyte (mg.)	Amount of nitrogen (μg.)	Proportion of nitrogen (μg./mg. dry wt.)	Mean dry wt. of single gametophyte (mg.)	Amount of nitrogen (μg.)	Proportion of nitrogen (μg./mg. dry wt.)	Mean dry wt. of single gametophyte (mg.)	Amount of nitrogen (μg.)	Proportion of nitrogen (μg./mg. dry wt.)
0·027	0·50	18·5	0·070	3·87	55·3	0·035	0·58	16·6	0·068	4·07	59·9
0·030	0·55	18·3	0·067	3·83	57·1	0·035	0·55	15·7	0·075	4·00	53·3
0·030	0·58	19·4	0·071	4·07	57·3	0·033	0·58	17·6	0·073	3·97	54·4

supplemented with 8-azaguanine alone and all new growth in these cultures had remained filamentous.

The amounts of protein nitrogen present in the gametophytes at the beginning and end of this experiment are given in Table 3. They show that when morphogenesis is restricted by the use of 8-azaguanine, the level of the protein nitrogen also remains depressed.

Discussion

The significance of the organic nitrogen content. In the present work the nucleic acids were not removed from the gametophytes before the estimation of the nitrogen, and consequently both the nucleic acids and protein will have contributed to the values obtained. The protein of the cell is known to be greater in amount than the nucleic acids. Hotta and Osawa (1958) estimated the protein nitrogen alone, the nucleic acids having first been removed from the gametophytes by trichloracetic acid. Subsequently Hotta, Osawa, and Sakaki (1959) estimated the amount of ribonucleic acid in gametophytes of *Dryopteris erythrosora* grown under the same conditions as those of Hotta and Osawa. Comparison of their results (reproduced logarithmically in Fig. 2) shows that during the first 60 days' growth the gametophyte contains from thirty to ten times as much protein as ribonucleic acid. During subsequent growth, in which the gametophyte enters the archegoniate condition, the proportion fluctuates, but the limits do not exceed those seen in the first period of growth. It is of interest to note that in the growing zone of the pea root, containing meristematic and differentiating cells, but lacking fully differentiated xylem and phloem, the ratio of protein to ribonucleic acid in the cell is of the same order as in the growing gametophyte, ranging from about twenty-five to almost ten (Heyes, 1960). The results of Heyes also show that the amount of deoxyribonucleic acid present is always considerably smaller than that of ribonucleic acid. Assuming quantitative relationship of this kind to hold for the cells of the gametophyte of *D. borreri*, it is clear that no great error will be introduced into the discussion of the nitrogen values of this species if they are taken as indicating protein alone. This view is supported by reference to Hotta and Osawa's results, from which it will be seen that comparable developmental stages of *D. erythrosora* and *D. borreri* contain very similar amounts of nitrogen per unit dry weight.

The rates of increase of the mass of the gametophyte and of the amounts of nitrogen present. The logarithmic growth-rate of the gametophyte of *D. borreri* appears to be more or less constant between the ages of 55 and 120 days (Fig. 3). Growth before the age of 55 days may also be exponential, but if so the logarithmic growth-rate is greater at this time than subsequently. If, however, the logarithms of the mean amounts of nitrogen present in single gametophytes are plotted against age, an almost exactly linear relationship is obtained (Fig. 3) for all the stages of growth from the spore to the emergence of the sporophyte. Although no estimations were made of the amounts of nitrogen in single gametophytes in the filamentous stages, the conformity of

the values obtained for the spore and cordate stages to the linear relationship points to the logarithmic rate of increase of the nitrogen having remained constant throughout the duration of the experiment. The view that filamentous stages are not exceptional is supported by the fact that if Hotta and Osawa's data for the amounts of nitrogen present in individual gametophytes

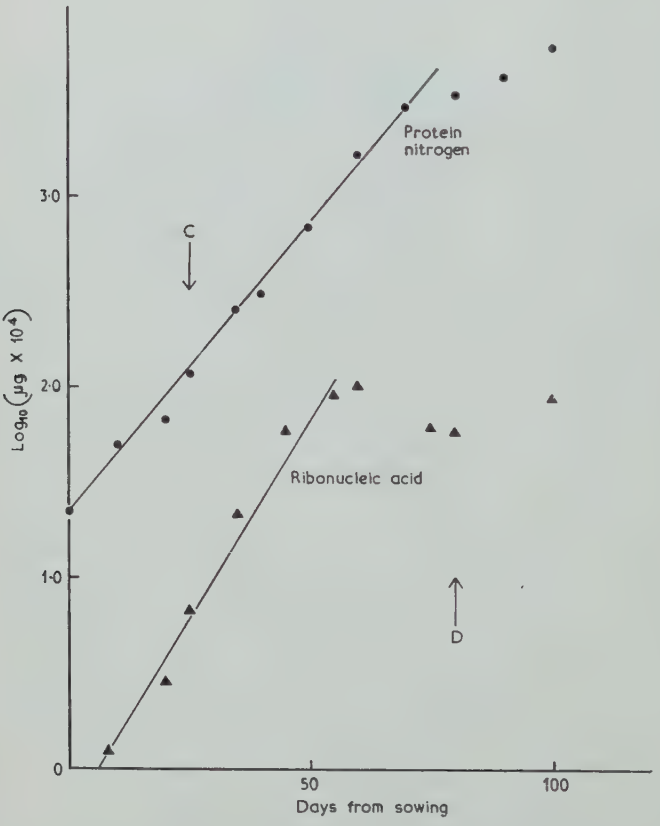

FIG. 2. The amounts of protein nitrogen and ribonucleic acid in gametophytes of *Dryopteris erythrosora* at different stages of growth. Data from Hotta and Osawa (1958) and Hotta, Osawa, and Sakaki (1959). C, appearance of cordate form; D, beginning of archegonia formation.

of *D. erythrosora* at different stages of growth (which included two filamentous stages) are plotted logarithmically against age (Fig. 2), a linear relationship is obtained for the first 70 days' growth. This is until some 45 days after the inception of the cordate form. Subsequent growth, which included the beginning of the production of archegonia, is accompanied by a fall in the logarithmic rate of increase of nitrogen.

These results show that in *Dryopteris erythrosora* up to the onset of oogenesis, and in *D. borreri* up to and including the initiation of the sporophyte

the increase in the protein is exponential, resembling the growth of an inoculum of unicellular organisms in a medium providing unlimited nutrition.

It is difficult to relate the results obtained from the second series of experiments, in which the organic nitrogen was estimated separately in the gametophyte and the sporophyte, directly with those from the first series.

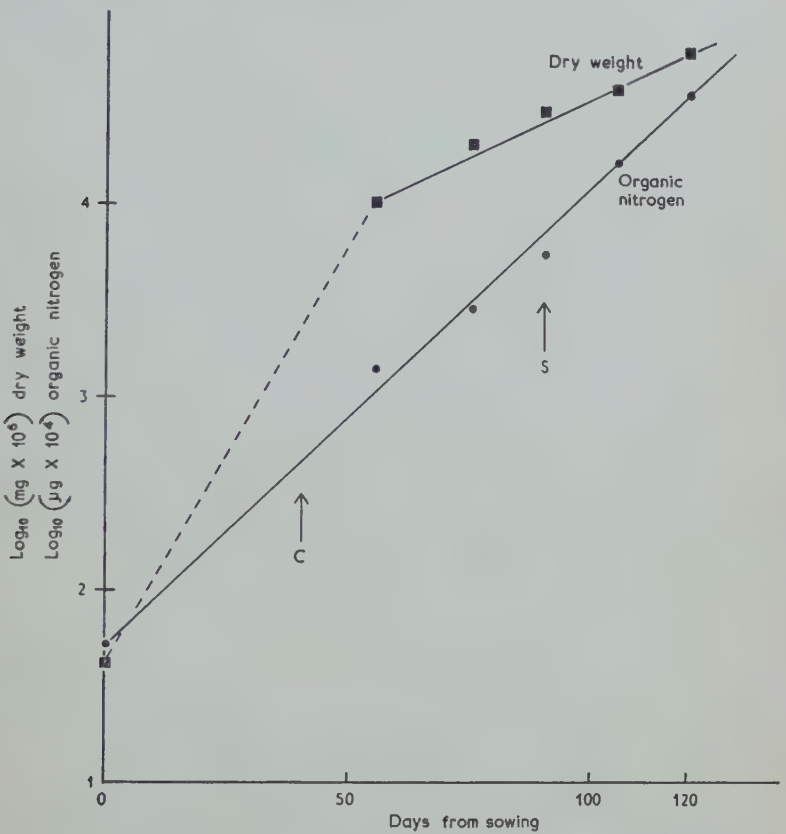

FIG. 3. The dry weights, $\log_{10}$ (mg. $\times 10^6$), of gametophytes of *Dryopteris borreri* at different stages of growth, and their content of organic nitrogen, $\log_{10}$ (μg. $\times 10^4$), plotted logarithmically. C, appearance of cordate form; S, initiation of sporophyte imminent.

A different sample of spores was used, and as Table 2 shows, in these gametophytes there was a different relationship between the dimensions of the sporophyte and those of the gametophyte. This may have been because these cultures grew at a greater rate, reaching in 105 days the dry weight (approximately 0·07 mg.) attained by the gametophytes of the first series in 120 days. The total nitrogen in the gametophyte and sporophyte taken together appears smaller than in gametophytes of equivalent development obtained at the end of the first series of experiments. This may be a consequence of the loss of

cytoplasmic material from the cells cut during the separation, especially since the cytoplasm is especially dense in the cells of the region where the cuts were made. The feature principally demonstrated by these estimations is how, once the growing-points of the sporophyte are laid down and the root, axis, and leaf primordium begin to extend, there is a rapid increase in the amount of non-proteinaceous material of the sporophyte, but a much less marked increase in the protein. During this extension the vascular tissue appears and the tracheids in particular will contribute to the non-proteinaceous component of the dry weight. It is interesting to note also from the results that, in the conditions in which the cultures were raised, the gametophyte goes on growing and accumulating protein after the initiation of the sporophyte.

The relationship between the synthesis of non-proteinaceous and proteinaceous materials during morphogenesis. The results of Hotta and Osawa (1958) and of the present investigation show that a change in the morphology of the gametophyte is accompanied by a change in the proportion of protein present (Fig. 1A). Hotta and Osawa were led to the view that in *D. erythrosora* a change in the morphology from filament to cordate form was accompanied, and possibly caused by, a rapid synthesis of protein. The re-examination of their results in the preceding section shows that they were misled; plotted logarithmically, the rate of increase of protein is constant and shows no fluctuations related to morphological change. The same is true of *D. borreri* (Fig. 3). Here the logarithmic rate of increase of protein is constant despite not only the change from filament to cordate form, but also the much more striking change from gametophyte to sporophyte.

The fluctuating proportion of protein during morphogenesis must therefore be re-interpreted. The changes in the proportions of protein in fact represent changes in the ratio of the non-proteinaceous to proteinaceous components of the plant. The non-proteinaceous component of the dry weight will consist principally of wall material, so fluctuations in the ratio will reflect changes in the synthesis of wall materials against a background of a constant rate of increase of protein. When the ratio of wall materials to protein falls, that is the proportion of protein rises, the accompanying morphological change is the formation in the gametophyte of a group of cells low in surface area (and therefore producing relatively little wall material), but rich in cytoplasm. These are in fact the meristems, firstly the apical meristem of the cordate gametophyte, and secondly in *D. borreri* the initial cells of the sporophyte.

The morphological effects of 8-azaguanine. It is of particular interest that in a certain range of concentration 8-azaguanine does not prevent growth, but halts or reverses the normal course of morphogenesis. Reference to Table 3 will show that the gametophytes supplied with 8-azaguanine increased their weight by about 17 per cent. This growth was at a reduced level of morphology and the synthesis of protein was hardly detectable. It seems likely that some synthesis of protein does go on in the presence of sub-lethal concentrations of 8-azaguanine, since the culture can remain growing for several weeks at least without necrosis. Nevertheless, the amount synthesized must be slight. This

is supported by Hotta and Osawa's results with *D. erythrosora*. Reference to their Fig. 2 will show that the proportion of protein nitrogen in filamentous gametophytes growing in the presence of 8-azaguanine falls at an increasing rate.

The results obtained with *D. borreri* indicate that the growth of *D. borreri* is very much more sensitive to the presence of 8-azaguanine than that of *D. erythrosora*. The appearance of the cordate gametophyte in *D. borreri* is prevented by a concentration of 8-azaguanine less than one-hundredth of that required to produce a similar effect in *D. erythrosora*. This difference seems too great to be accounted for by differences in the purity of the materials used. Apart from this, the qualitative effect of 8-azaguanine on the early stages of growth in *D. borreri* is the same as in *D. erythrosora*, with the additional effect in *D. borreri* that 8-azaguanine prevents the initiation of the sporophyte when it is supplied to cordate gametophytes previously undergoing active growth. The later experiments of Hotta, Osawa, and Sakaki (1959), in which the gametophytes were supplied with radioactive azaguanine, strongly suggest (but do not prove conclusively) that 8-azaguanine is incorporated into the ribonucleic acid of gametophytes of *D. erythrosora*, as it is into the ribonucleic acids of other organisms supplied with it. It can therefore be accepted that 8-azaguanine enters into the ribonucleic acid of the *D. borreri* gametophyte, and that a gametophyte containing this abnormal ribonucleic acid is incapable of further morphogenesis.

The relationship between the synthesis of ribonucleic acid and morphogenesis. Hotta, Osawa, and Sakaki (1959) estimated the amounts of ribonucleic acid in gametophytes of *D. erythrosora* at different stages of growth. They concluded that the transition to the cordate form was accompanied by a very rapid synthesis of this nucleic acid, although the increase in the synthesis began before the morphological transition became apparent. It thus preceded the similar rise in the synthesis of protein claimed by Hotta and Osawa. If, however, the curve given by Hotta, Osawa, and Sakaki for the amounts of ribonucleic acid in the gametophyte of *D. erythrosora* is replotted logarithmically it shows that up to at least 40 days after germination the rate of increase of ribonucleic acid is almost exactly exponential (Fig. 2), similar to that of the protein. Indeed, this is to be expected if there is, as discussed earlier, a close relationship between the amounts of ribonucleic acid and protein in systems of growing cells. There is, therefore, in *D. erythrosora* no convincing evidence that the logarithmic rate of increase of the ribonucleic acid changes at any time in relation to morphogenesis during the development of the cordate gametophyte from the spore, although a marked fall in the rate begins shortly after the cordate gametophyte has been formed. This fall precedes by about 20 days the similar fall in the rate of increase of protein in this species.

In *D. borreri*, however, since the logarithmic rate of increase of protein remains constant until the emergence of the sporophyte (Fig. 3), there is the interesting possibility that there is an accompanying steady rise in the amount of ribonucleic acid. This would imply that the factors governing the rate of

production of the ribonucleic acid suffered no change during the initiation of the sporophyte and possibly during the early stages of its emergence, so that the amount of ribonucleic acid continued to increase exponentially. In *D. erythrosora*, on the other hand, the factors governing the rate of production of the ribonucleic acid in the gametophyte taken as a whole appear to change, to the detriment of the rate, as the gametophyte enters the archegoniate condition. These differences might be causally related to differences in the morphogenesis of the two species. In *D. erythrosora* the archegoniate condition is of indefinite duration and, in the absence of fertilization, represents a halt in morphogenetic advance. In *D. borreri* there is no such interruption and in the conditions of culture *in vitro* the progression from spore to sporophyte is a continuous process.

Thus, although in *D. erythrosora* a check in the morphogenetic advance of the gametophyte, resulting from the processes of sexual reproduction, may be related to a *depression* in the net rate of synthesis of ribonucleic acid, there is no evidence in either *D. erythrosora* or *D. borreri* (assuming the steady rate of the synthesis of protein in this species to indicate a similar steady rate in the synthesis of ribonucleic acid) that the morphological transitions occurring in continuous development can be related to *increases* in the net rate of synthesis of this nucleic acid.

The cause of morphological change. Although there is no evidence of increases in the net rates of synthesis of protein or ribonucleic acid occurring in relation to morphological change in the gametophyte, increases in the rates within the cells initiating the new level of morphology cannot be ruled out. This possibility must be allowed since, although an exponential curve will follow if each unit of protein increases by a constant amount in unit time, the existence of an exponential curve is in itself no evidence that the protein within the cells actually increases in this way. It is known that the amount of protein increases in enlarging and vacuolating cells of the pea root (Heyes, 1960), but in the cordate gametophyte the cells of the posterior region undergo no further visual change with age and may have ceased to synthesize protein. The number of these fully differentiated cells increases with age. Thus, although the net rate of increase of protein in the gametophyte is exponential, the actual rate of production of new protein per unit of protein may not be constant in the meristematic and differentiating cells, but increase. The same argument applies to the ribonucleic acid. These questions cannot be decided on the evidence available.

There is firm evidence in both *D. erythrosora* and *D. borreri* of changing rates of synthesis of non-nitrogenous materials during the development of the gametophyte, leading to changing quantitative relationships between these different components of the living system. These changing relationships are reflected in the morphology, but are not themselves the cause of the morphological transitions, since at each transition there are changes not only in the synthesis of wall materials, but also in the behaviour of the cytoplasm. The meristematic cells of the cordate gametophyte and the initial cells of the

sporophyte do not, for example, contain large vacuoles as do the other cells of the gametophyte. Although, as we have seen, the possibility that changes in cell behaviour of this kind, leading to new anatomical and morphological features, are associated with quantitative changes in the nucleic acid and protein present cannot be excluded, such evidence as there is points rather to the quality of these substances as being of importance in morphogenesis. Hotta, Osawa, and Sakaki (1959), for example, have detected differences in the composition of the ribonucleic acids present in filamentous and cordate forms respectively of the gametophytes of *D. erythrosora*. In pea roots the analyses reported by Heyes (1960) suggest a lower proportion of purines in the ribonucleic acid of the cells of the tip than in that of the cells of the differentiating region behind. In seedlings of wheat Wright (1960), using a serological technique, has shown that the proteins of the coleoptile are not identical with those of the seed or root. There is, however, a close similarity with those of the leaf, an organ of similar nature. Evidence of this kind indicates a qualitative diversification of the ribonucleic acid and protein during growth and development related to the anatomy and morphology of the organs generated. The known relationship between the presence of ribonucleic acid and the synthesis of protein suggests that the diversification of the former is of prime importance in the biochemistry of morphogenesis.

There are a number of ways in which changes in the behaviour of cells and in the kind of growth might follow from an increase in the variety and relative quantities of the proteins present in the meristematic regions. Many of these proteins, for example, will be functioning as enzymes, and consequently new metabolic processes concerned with growth might arise and others change in relative rate. Also, increasing complexity of the proteins in a cell might allow a greater number of possible molecular configurations in the cytoplasm, and this in turn influence the number of possible orientations of the spindle at mitosis. This would permit striking changes in morphology, of the kind seen in the transition from the filamentous gametophyte, where growth is one-dimensional, to the cordate, where initially it is two-dimensional. The change from the cordate gametophyte to the sporophyte is again of this nature, namely from complanate, largely two-dimensional, growth to growth involving cell division in numerous directions.

The significance of the results in the interpretation of the life cycle of the Polypodiaceous ferns. The striking feature of the development of *D. borreri* is the absence of any check in morphogenesis from the germination of the spore to the emergence of the sporophyte. This is correlated with an exponential increase in the protein present, and possibly also of the ribonucleic acid. In *D. erythrosora* in the absence of fertilization the gametophyte remains indefinitely in the archegoniate condition. This pause in morphogenesis is correlated with a falling off in the rate of synthesis of protein and ribonucleic acid. In a fern reproducing sexually, only the egg cell is normally capable of giving rise to the sporophyte. In *D. borreri*, however, and probably in all other obligately apogamous ferns (see, for example, Duncan, 1943), it is not

possible to identify any one cell as the initial of the sporophyte; the sporophyte first becomes identifiable as a group of cells. So far as could be detected in *D. borreri*, none of these cells, nor any of their antecedents, showed any of the features of egg cells. In none, for example, was there an expansion of the nucleus and an accompanying loss of chromaticity, such as occurs in the eggs of several Polypodiaceous ferns (Vazart, 1952; Bell, 1959). Also autoradiographs, prepared by the same procedure as that followed by Bell (1961) in *Pteridium*, gave no evidence of an interaction of nucleus and cytoplasm similar to that occurring in the egg of *Pteridium* in any of the cells of that region of the gametophyte of *D. borreri* which gives rise to the sporophyte. Thus in *D. borreri* the transition to the sporophyte seems to result from a change in the properties of a group of cells in the sub-apical region, this change occurring progressively during growth and development. Preparation for the morphological transition is not confined to one cell, nor associated with any distinctive process such as oogenesis.

If qualitative as well as quantitative changes in the ribonucleic acid and protein are essential for increasing morphological complexity, then in a fern reproducing sexually the changes responsible for the difference in morphology of the two generations of the life cycle must occur principally in the egg cell. The intimate interaction of nucleus and cytoplasm in the egg of *Pteridium* (Bell, 1961) might provide a means by which these changes are brought about. An egg cell in the ferns, however, rarely if ever develops further without fertilization, so the transformation of the contents of the cell must exhaust the potentialities of the cell for further growth, unless fertilization occurs. This, in effect, is the introduction into the cell of a compact nucleus which may act as a template stimulating the reorganization of the nuclear material of the egg and allowing further growth. It appears, therefore, that oogenesis is a process of major biochemical importance, during which quantitative and qualitative changes take place in the ribonucleic acid and protein of the egg cell, so that all subsequent growth from it acquires a more complex morphology.

In *D. borreri*, where nothing comparable to oogenesis occurs, similar biochemical changes must occur directly in the sub-apical cells. These cells correspond in position to those producing archegonia in sexually reproducing ferns, suggesting that the metabolism of the cells in this region has special characteristics. Occasionally in sexually reproducing ferns the morphological transition to the sporophyte can take place directly from the somatic cells of this region of the gametophyte. The frequency with which this occurs can sometimes be increased by supplying the gametophytes with glucose (Whittier and Steeves, 1960), but it still remains a rare event. This rarity suggests that the biochemical changes normally accompanying oogenesis can occur, but only as an irregularity, in the somatic cells of the gametophytes of sexually reproducing ferns. On the other hand, similar changes leading to the appearance of a more complex morphology must occur readily, unaccompanied by any distinctive cytological process such as oogenesis, in the sub-apical region

of the gametophyte of *D. borreri*. In ferns such as these archegonia are never formed, and reproduction is always apogamous. Since in *D. borreri* the biochemical changes associated with the transition from the gametophyte to the sporophyte seem to be a continuation of those occurring in the preceding morphogenesis of the gametophyte, there is the interesting possibility that obligately apogamous reproduction will admit of a less complex resolution in terms of the physiology of growth than normal sexual reproduction.

Summary

1. The development in pure culture of the gametophytes of *D. borreri*, a fern reproducing apogamously and lacking archegonia, was followed, and a number of stages of growth were defined.

2. The mean amounts of nitrogen present at each stage of growth were estimated. The increase in the amount of nitrogen during growth, including the initiation and emergence of the sporophyte, was found to be exponential. The organic nitrogen was taken to represent principally protein.

3. The proportion of organic nitrogen in the gametophytes rose at the transition from the filamentous to the cordate form, and again at the initiation of the sporophyte. These rises are shown to be caused by changes in the rates of synthesis of non-nitrogenous materials.

4. It is shown from the results of earlier workers that the amount of protein in the developing gametophytes of *D. erythrosora*, a fern reproducing sexually, also increases exponentially, but that the logarithmic rate of increase falls as the gametophyte enters the archegoniate condition. The increase in the ribonucleic acid in the gametophytes of this fern is also shown to be exponential in the first stages of growth, the logarithmic rate of increase falling some 20 days before that of the protein.

5. The results are considered in relation to the life cycle of the Polypodiaceous ferns. It is concluded that qualitative and possibly quantitative changes in the ribonucleic acid and protein are involved in the transition from gametophyte to sporophyte. It is also suggested that, since in the gametophyte of *D. borreri* these changes do not appear to be checked and confined to a single cell, as in *D. erythrosora*, apogamous reproduction may be easier to explain physiologically than sexual.

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Life Histories in the Algae

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With three Figures in the Text

ABSTRACT

The earlier schemes of algal life-history classification are critically discussed and a new and fuller scheme of classification is proposed. It is based upon (*a*), the cytological alternation as generally recognized, and (*b*), morphological alternation described in terms of individuals, generations, and the morphological similarity or dissimilarity of the latter. Two completely new groups, the Polyplont and Polybiont, are proposed.

In conclusion, a probable course of life-history phylogeny is outlined.

OUR knowledge of algal life histories has been greatly extended, especially in the Chlorophyceae and Rhodophyceae, since this subject was discussed by Drew in 1955. Whilst the basic background remains the same it has not proved easy to integrate some of the newly found life histories with the schemes of previous workers. The present authors believe that a new scheme is therefore desirable and it is with this in view that the present contribution has been written. Previous reviewers of this subject have generally confined their attention to the three major divisions of the algae—the Chlorophyceae, Phaeophyceae, and Rhodophyceae—and we propose to do the same here. Any system of classification should describe as accurately and as completely as possible the entire life history of any plant in the group under consideration. It must be such that confusion between types is not possible and it should also be applicable to all known species. In many of the algae, especially in the Phaeophyceae (Ectocarpales, Dictyosiphonales, Chordariales) and Rhodophyceae (e.g. polyploids of *Spermothamnion turneri* and *Plumaria elegans*), it is no longer possible to refer to an obligatory life cycle. In view of this we have followed Drew (1955) and replaced the term *life cycle* by *life history*.

In so far as Drew in 1955 has given a survey of the earlier literature on this subject it is considered unnecessary to repeat this in any detail. It is convenient, however, to outline briefly the different approaches that have been taken by previous writers.

The modern treatment of algal life histories stems from Svedelius's contribution in 1931, wherein he classified the various types of algal life history on the basis of the *nuclear* phases. He established the following groups:

[Annals of Botany, N.S. Vol. 25, No. 100, 1961.]

(*a*) *haplonts*: algae in which only the zygote is diploid and reduction division takes place at its germination.

(*b*) *diplonts*: algae in which only the gametes are haploid and reduction division takes place at gametogenesis.

(*c*) *diplohaplonts*: algae in which there is an alternation of diploid and haploid generations.

In addition he found it necessary to incorporate two earlier terms (Svedelius, 1915) which initially had been proposed for certain Rhodophyceae:

(*d*) *haplobiont*: algae with only one kind of individual or biont in the life cycle. The generation can be either haploid, e.g. *Scinaia*, or diploid, e.g. *Codium*, *Fucus*. No distinction is made between separate male and female plants which are regarded as the same individual.

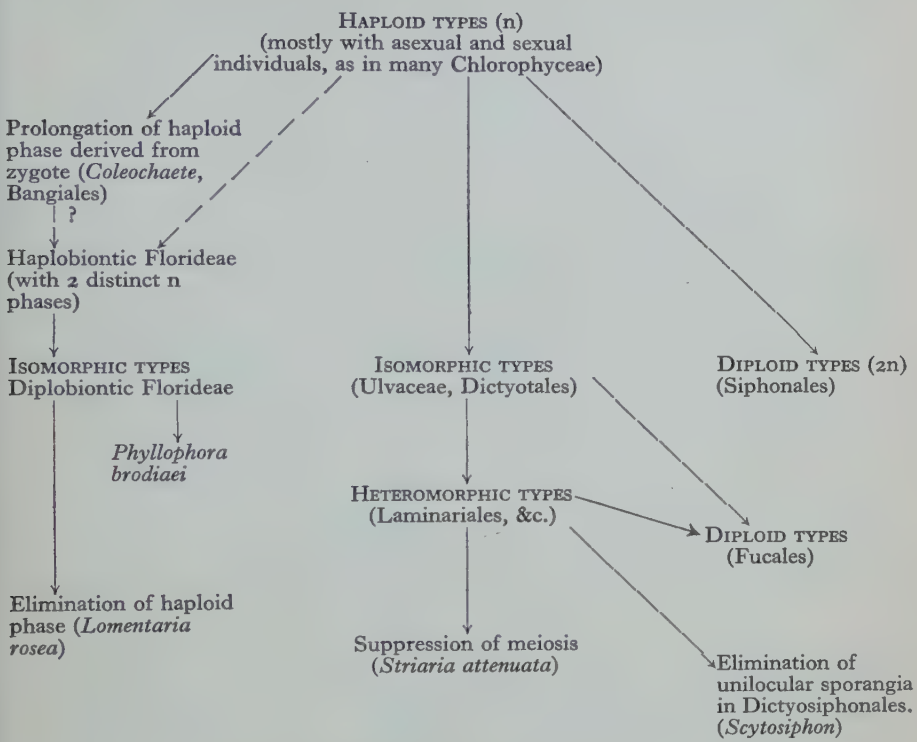
(*e*) *diplobiont*: algae with two kinds of individual or biont in the life cycle, e.g. *Polysiphonia*.

This classification, however, did not really provide an adequate niche for those members of the Rhodophyceae with haploid or diploid carposporophytes, nor did it provide any indication whether diplobionts had an isomorphic or a heteromorphic alternation.

In 1938 Kylin accepted Svedelius's diplo- and haplobionts as representative of the Rhodophyceae. In the Phaeophyceae, however, Kylin recognized three types of life history which he used as a basis for his classification. The Isogeneratae included algae with alternation of morphologically similar generations, e.g. *Dictyota*; the Heterogeneratae comprised genera with alternation of dissimilar generations, and they were subdivided according to whether the gametophyte, e.g. *Cutleria*, or the sporophyte, e.g. *Laminaria*, was dominant; the Cyclosporeae contained the Fucales in which only the diploid generation was present. In the same year Smith (1938) recognized these same three types of Phaeophycean life history but did not make the same subdivision as Kylin between the *Cutleria* and *Laminaria* types in the Heterogeneratae. In the Chlorophyceae both workers considered that there were three basic types of life history, e.g. algae with a haploid generation only (*Ulothrix*), algae with a diploid generation only (*Codium*), and diplobionts, e.g. *Ulva*, *Cladophora* spp. Smith (1938) realized the necessity of dividing the Rhodophyceae into those with a biphasic alternation of generations (haploid gametophyte and haploid or diploid carposporophyte but with no tetrasporophyte) and those which are triphasic (haploid gametophyte and diploid carposporophyte and tetrasporophyte). Those with biphasic alternation he subdivided according to whether the carposporophyte was haploid or diploid. This represented an advance on Kylin's classification.

In 1942 Fritsch recognized four major types of algal life history, though he was careful to state that there were deviations in a small number of species. These four major types were the haploid (= haplobiontic haplont of Svedelius), diploid (= haplobiontic diplont), isomorphic (= isomorphic diplobiont or Isogeneratae of Kylin) and heteromorphic (= heteromorphic diplobiont or Heterogeneratae of Kylin). The main types and the derivations together with

their conjectured evolutionary development are illustrated in the scheme below.



In the light of present-day knowledge this scheme, if it is accepted, is in need of revision. Among the Chlorophyceae there are heteromorphic types, e.g. *Halicystis/Derbesia*, *Spongomorpha/Codiolum*, *Codiolum polyrhizum*, and diploid types represented by the Siphonales, which may well have been derived from isomorphic types as found in the Siphonocladales (Chapman, 1954).

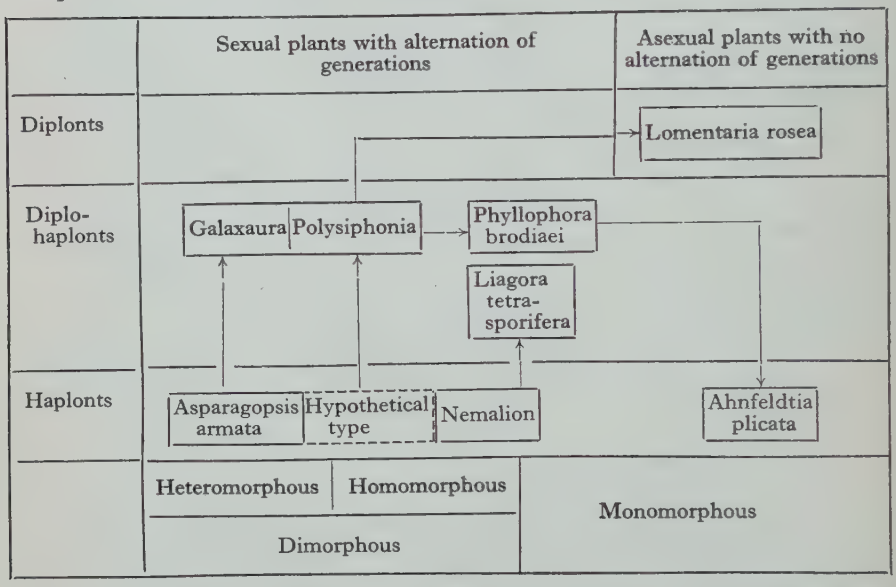
In 1945 a very interesting paper by Hygen appeared which, rather surprisingly, was ignored by later writers on this subject (Chadefaud, Feldmann, Drew). Hygen redefined Svedelius's terms of haplont, diplont, and diplohaplont as follows:

Haplont: a plant which is haploid throughout the entire vegetative stage.
Diplont: a plant which is diploid throughout the entire vegetative stage.
Diplohaplont: a plant whose vegetative stage comprises both a haploid and diploid phase.

Because the terms 'haplobiont' and 'diplobiont' have not been always correctly used in the literature, Hygen proposed that they be abandoned and replaced by the following new terms:

1. *Monomorphous*: plants with only one type of individual[1] in the life history.
2. *Dimorphous*: plants with two types of individual with different reproductive organs in the life history. Male and female plants are not regarded as distinct individuals. Two subtypes are recognized:
   (*a*) Homomorphous: plants in which the two types of individual are morphologically alike.
   (*b*) Heteromorphous: plants in which the two types of individual are morphologically unlike.

Hygen concluded by summarizing these types in a table and suggested their possible interrelationships (see below).



In 1952 Chadefaud pointed out that there is both a morphological and a cytological aspect to the sexual cycle. Using this approach as a basis, he classified the algae into three categories:

(*a*) Algae with isomorphic, e.g. *Ulva*, or heteromorphic, e.g. *Laminaria*, alternation.

(*b*) Algae with 'gamontes' (gamete-producing plants) only in the life history. The 'gamontes' can be either haploid or diploid, e.g. *Fucus*.

(*c*) This group he subdivided as follows:
   (i) The normal diplobiontic Florideae with three generations in the life history.
   (ii) *Spermothamnion turneri* with sex organs on both haploid and

[1] Hygen does not regard the individual as necessarily synonymous with a generation. This is an important distinction.

diploid plants, so that there may be two sexual cycles, one haploid and diploid and the other diploid and tetraploid.

(iii) Species of *Ectocarpus* in which the diploid plants function as both 'sporontes' and 'gamontes'.

In the same year (1952) and using the same approach—namely the independence of the morphological and cytological phases—Feldmann proposed a new terminology:

Cytological cycles

1. Meiosis occurs at germination of the zygote. The generation is a ***haplont*** and the cycle is ***haplophasic.***
2. Meiosis occurs at gametogenesis. The generation is a ***diplont*** and the cycle is ***diplophasic.***
3. The cycle exhibits an alternation of two phases (***diplohaplophasic***), the plant arising from the zygote being a ***diplont*** and that arising from a spore being a ***haplont.***

Morphological cycles

1. Algae with one generation only (haploid or diploid)—*Monogenetic.*
2. Algae with two generations, either isomorphic or heteromorphic—*digenetic.*
3. Florideae with three types of morphological generation, all haploid (Bonnemaisoniales) or two diploid and one haploid—*trigenetic.*

This classification can be demonstrated most conveniently by the following table.

Morphological Alternation

		Monogenetic	Digenetic			Trigenetic
			Isomorphic	Heteromorphic		
				Gametophyte dominant	Sporophyte dominant	
Cytological Alternation	Haplophasic	*Chlamydomonas* Zygnemales Charophyta		Haplobiontic Florideae		Bonnemaisoniales
	Diplohaplophasic		*Ulva* *Cladophora* *Zanardinia* Dictyotales	*Cutleria* *Phyllophora brodiaei* *Liagora tetrasporifera*	Laminariales Chordariales	Diplobiontic Florideae
	Diplophasic	*Codium* *Fucus*	*Cladophora glomerata*			

The most recent contribution has been that of Drew (1955). She commences from the basis that life histories in the algae must be considered in terms of both morphology and cytology but that, in addition, one must include any seasonal or regional variations. Drew prefers the use of the term *life history* rather than life cycle, sexual cycle, &c. and she defines it as 'the recurring

sequence of somatic and nuclear phases characteristic of the species under discussion'. The term 'somatic phase' applies to any phase with constant morphological characters. So far as nuclear phases are concerned, Drew recognizes two groups only:

1. *Monophasic* algae, which equates jointly the haplonts and diplonts of Svedelius or the haplo- and diplophasic groups of Feldmann. These algae will be all wholly haploid or diploid in their vegetative state.
2. *Diphasic* algae, which equates the diplohaplonts of Svedelius and the diplohaplophasic group of Feldmann. Drew restricts the use of the terms 'iso-' and 'heteromorphic' to qualifying these diphasic algae.

Drew (loc. cit.) defines a somatic phase as 'a state of an organism recognizable by a constant characteristic morphological appearance independent of the chromosome number'. According to the number of somatic phases that are exhibited, the life history of a species can be termed mono-, di-, or trimorphic. Species which possess an alternation of two morphologically similar generations are regarded as monomorphic. In the Chordariales (Phaeophyceae) where there may be two kinds of dwarf filament, one gametophytic (prothallial) and the other diploid (protonemal), the life cycle is regarded as trimorphic, though this seems difficult to justify in the light of the treatment accorded to isomorphic diplohaplonts. In the Rhodophyceae the isomorphic sexual and asexual plants are regarded as one somatic phase but the carposporophyte is treated as a separate one. In *Bonnemaisonia asparagoides* and *Asparagopsis armata* three haploid somatic phases are recognized, and also in *Batrachospermum* where the 'Chantransia' stage is regarded as a separate somatic phase rather than a juvenile condition. On the above basis Drew exemplifies her somatic phases in the following scheme:

	Chlorophyceae	*Phaeophyceae*	*Rhodophyceae*
Monomorphic	*Codium* *Ulva* *Monostroma*	*Fucus* *Dictyota* *Laminaria*	
Dimorphic	*Halicystis/Derbesia*		*Nemalion* *Polysiphonia*
Trimorphic		Some ? Chordariales	*Batrachospermum* *Bonnemaisonia*

Monostroma is regarded as dimorphic because the zygote, although not undergoing division, does enlarge and food material accumulates. Similar hypnozygospores are found in other orders of the Chlorophyceae. Drew considered that the somatic phases are very much more important than nuclear phases in distinguishing between types of life history. She recognizes the following types:

1. *Monomorphic diplohaplonts*: -A-a-A-a-[1]

   Ulva, *Enteromorpha*, *Cladophora* species, *Dictyota*, *Ectocarpus siliculosus*.

[1] Capital letter = diploid phase, lowercase = haploid. A second letter (B, b) represents a second, different somatic phase.

2. *Monomorphic diplonts*: -A-A-A-
   Siphonales, Fucales.

3. *Dimorphic diplohaplonts*: -A-b-A-b- or -a-B-A-a-B-A-
   Halicystis/Derbesia, Laminariales, *Polysiphonia*.

4. *Dimorphic haplonts*: -a-a-B-a- or -a-b-a-b
   Chlamydomonas spp., *Volvox aureus*, *Nemalion*.

5. ? *Trimorphic diplohaplonts*: -A-b-C-A-
   ? Some Chordariales, Dictyosiphonales.

6. *Trimorphic haplonts*: -a-b-c-a-b-c
   Batrachospermum, some Bonnemaisoniaceae.

Apart from the validity of group 5, the first example in type 4 (-a-a-B-a-) raises doubt as to whether it is correctly assigned or whether it should not really be regarded as a dimorphic diplohaplont. It depends on the status given to the morphological diploid phase represented by the persistent or enlarging hypnozygospores. No adequate provision is made in the classification for examples such as *Liagora tetrasporifera* and *Helminthocladia hudsoni*, though they are listed by Drew without status. She also considered that it was not possible adequately to classify *Ahnfeldtia plicata* and *Gymnogongrus griffithsiae*. The present authors are, however, of the opinion that an adequate classification of life histories is possible, though they realize that further work in the future may render modification necessary and possibly even the introduction of new types.

Indications have already been given that the schemes briefly outlined above possess defects. In the Svedelius scheme the terms embraced too wide a range of algal life histories and no cognizance was taken of the morphological cycles. In Fritsch's scheme again too wide a range of life histories was included in a group and he took no account of the nuclear phases, preferring to rest his classification solely upon morphological criteria.

Hygen redefined Svedelius's terms. He realized the importance of nuclear and morphological cycles and characterized them in a grid system. Although he appreciated the difference between individuals and generations, he did not include this in his classification. Like the previous workers his morphological divisions were too large and should have been further subdivided.

Feldmann was the first to subdivide the morphological phases into workable units, but, in view of more recent knowledge and because of some complex types, we believe further subdivision is desirable. We also consider his terminology for the morphological phase, using the suffix 'genetic', to be an unfortunate choice, since this term is usually connected with cytological phenomena.

Drew's classification suffered from the disadvantage that it did not conveniently accommodate some members of the Nemalionales, e.g. *Liagora tetrasporifera*, *Helminthocladia hudsonii*, and she failed to subdivide morphological cycles beyond the mono-, di-, and trimorphic cycles. On the basis of

these criticisms we have devised a new scheme of classification. We have followed to some extent each of the earlier workers in this field, but we differ from them in certain points:

1. Morphological phases are treated rather more fully.
2. We have distinguished between morphological individuals and generations; this need arises primarily from a consideration of the Floridean carposporophyte.
3. We have included an extra morphological and cytological class to overcome the apparent difficulty of polyploid series.

It is necessary now to establish the criteria upon which a further classification of life cycles can be based. It is clear that both morphological and cytological phases are to be distinguished, and that in general it is agreed that the morphological are the more important. In the case of the Florideae, however, the disposition of the carposporophyte has to be considered. Morphologically this is borne on the female gametophyte, but since it arises from the zygote it is regarded as a separate generation. A generation is therefore regarded as distinct from a morphological individual.

Morphological alternation

This we define as alternation between separate individuals or *bionts* that are morphologically similar or dissimilar. Following Hygen (1945) we define an *individual* or *biont* as *a completely separate unit or phase derived from the preceding individual by free gametes or spores*. This definition excludes the carposporophyte of the Rhodophyceae and also the sexual appendages of *Prasiola stipitata* (Friedmann, 1959) as separate individuals. We do not regard male and female plants as separate individuals under this terminology.

Three kinds of morphological alternation can be distinguished in the algae: these are *monobiontic* with one kind of individual or biont; *dibiontic* with two kinds of individual or biont; *polybiontic* with more than two kinds of individual or biont. This last category is a new departure from schemes of previous writers and is devised to include polyploid series, e.g. *Spermothamnion turneri*, even though the individuals may be independent genetically and not necessarily giving rise to other members in the polyploid series. This category will also include *Plumaria elegans*, which has an independent triploid generation as well as the normal haploid and diploid.

Further morphological distinction is obtained by considering the number of morphological generations present in the life history. Again following Hygen we define a *generation* as *a vegetatively developed phase of the life cycle commencing with germination or enlargement of spores or zygotes and terminating in gametogenesis or sporogenesis, or in the development of a completely different morphological form by budding, and not necessarily independent of the preceding phase*. This definition makes the Rhodophycean carposporophyte a separate generation, as also the leafy haploid lobes of *Prasiola stipitata*. It will be

appreciated that morphologically the generations correspond to cytological phases whereas the individuals or bionts do not. The morphological generations are divided into groups as follows:

Monogenic, with one generation only in the cycle.
Digenic, with two generations in the life cycle.
Trigenic, with three generations in the life cycle.
Polygenic, with more than three generations in the life history, e.g. *Spermothamnion turneri*. *Plumaria elegans* with only haploid, diploid (including carposporophyte), and triploid plants can be termed tetragenic.

The algae with digenic life cycles are further subdivided according to whether the generations are isomorphic or heteromorphic. Following Feldmann, we consider that the heteromorphic life cycles can be further classified on the basis of dominance of either the gametophyte or the sporophyte generation. Algae such as *Halicystis*/*Derbesia* and *Codiolum polyrhizum* we regard as having both generations equally represented. Algae with trigenic life histories are subdivided according to whether two (dimorphic) or three (trimorphic) distinctly different morphological phases are present. *Polysiphonia* is typical of the former and plants such as *Asparagopsis armata* of the latter.

Cytological alternation

Here we have followed the example of previous writers on the subject and we retain the terms *haplont*, *diplohaplont*, and *diplont* as defined by Feldmann (1952), who based his definitions upon the place of the reduction division in the cycle. For those species with three or more cytological generations, e.g. *Plumaria elegans* and *Spermothamnion turneri*, we have added a fourth category which we term *polyplont*. It has been argued in the past (Drew, 1955) that these polyploids, and in particular the triploid phase of *Plumaria elegans*, have the different phases genetically independent and that therefore they are anomalous and should not be included in any scheme.

The classification that is proposed here is not based upon any obligatory life history but rather on a consideration of all the possible stages that the plants can show. On this basis we feel justified in including the polyplont category.

Haplont: plant in which all generations are haploid, reduction division occurring at zygote germination.
Diplohaplont: plant with both diploid and haploid generations, reduction division taking place at sporogenesis.
Diplont: plant in which all generations are diploid, reduction division occurring at gametogenesis.
Polyplont: plant with triploid or tetraploid generations in addition to the normal haploid and diploid, the extra generations apparently reproducing only by spores.

It may appear that certain plants are excluded under this classification, e.g. *Ahnfeldtia plicata*, *Lomentaria rosea* in European waters. The former can,

however, be legitimately treated as a haplont in which no reduction division ever occurs, and the latter as a diplont in which no reduction division ever occurs. They can be specified as an ameiotic haplont and ameiotic diplont respectively.

The above classification is summarized in the table on p. 557.

In conclusion we give below some illustrations of the way in which the life histories of different species are classified on the various systems.

Scinaia: Haplobiontic haplont (Svedelius)
Monomorphous haplont (Hygen)
Heteromorphic digenetic haplophase (Feldmann)
Dimorphic haplont (Drew)
Heteromorphic digenic dibiontic haplont (Chapman and Chapman).

Bonnemaisonia: Diplobiontic haplont (Svedelius)
Heteromorphous haplont (Hygen)
Trigenetic haplophasic cycle (Feldmann)
Trimorphic haplont (Drew)
Trimorphic trigenic dibiontic haplont (Chapman and Chapman).

Polysiphonia: Diplobiontic diplohaplont (Svedelius)
Homomorphous diplohaplont (Hygen)
Trigenetic diplohaplophasic cycle (Feldmann)
Dimorphic diplohaplont (Drew)
Dimorphic trigenic dibiontic diplohaplont (Chapman and Chapman).

Codium: Haplobiontic diplont (Svedelius)
Monomorphous diplont (Hygen)
Monogenetic diplophasic cycle (Feldmann)
Monomorphic diplont (Drew)
Monogenic monobiontic diplont (Chapman and Chapman).

In the Chlorophyceae we believe the monobiontic, monogenic haplont represents the primitive type of life history. With the commencement of a delay in the reduction division, e.g. *Monostroma* and hypnozygosporous Chlamydomonadaceae, this primitive life history developed into a monobiontic, digenic, diplohaplont type. Further delay in the reduction division produced the dibiontic, digenic, isomorphic diplohaplont life history of which the dibiontic, digenic, heteromorphic diplohaplontic type can be regarded as a variant. Finally the elimination of the sexual haploid generation led to the final type—the monobiontic, monogenic diplont (Fig. 1).

In the Phaeophyceae we are of the opinion that the primitive life history is the dibiontic, digenic, isomorphic diplohaplont. The monobiontic, monogenic haplont life history of *Ectocarpus virescens* is probably a reduced type rather than a primitive one. The basic life history evolved to a dibiontic, digenic, heteromorphic diplohaplontic type in which either the gametophyte or sporophyte generation could be dominant, the former perhaps being

Morphological Cytological	Monobiontic		Dibiontic					
	Monogenic	Digenic	Digenic				Trigenic	
			Isomorphic	Heteromorphic			Dimorphic	Trimorphic
				Gametophyte dominant	Neither dominant	Sporophyte dominant		
Haplont	Conjugales Ameiotic *Ahnfeldtia plicata*	*Scinaia furcellata* *Nemalion multifidum*		*Porphyra?*				*Batrachospermum moniliforme* *Asparagopsis armata* *Bonnemaisonia hamifera*
Diplohaplont	?	*Prasiola stipitata* *Monostroma* *Phyllophora brodiaei* *Liagora tetrasporifera* *Helminthocladia hudsonii* Drew pp. 365–6, 372	*Enteromorpha* *Ulva lactuca* *Cladophora* *Dictyota dichotoma* See Drew pp. 363, 368	*Cutleria multifida* *Spongomorpha*	*Halicystis/Derbesia* *Codiolum polyrhizum* *Enteromorpha prolifera*	Laminariales *Desmarestia* *Splachmidium* Drew pp. 369–70	*Polysiphonia violacea* Most Florideae? Drew p. 373 Dictyosiphonales? Chordariales?	*Nemalion helminthoides* *B. asparagoides*
Diplont	*Codium* *Valonia utricularis* *Fucus* *Bryopsis plumosa* *Caulerpa prolifera* Drew pp. 365, 369 Ameiotic *Lomentaria rosea*		*Cladophora glomerata*					

Note. Some of the examples given have not been proved definitely, but are, rather, very probable cases. Thus the monobiontic and trigenic Caepidium is probably a diplohaplont. In addition there are the polybiontic polyplonts, *Plumaria elegans* (tetragenic) and *Spermothamnion turneri* (polygenic).

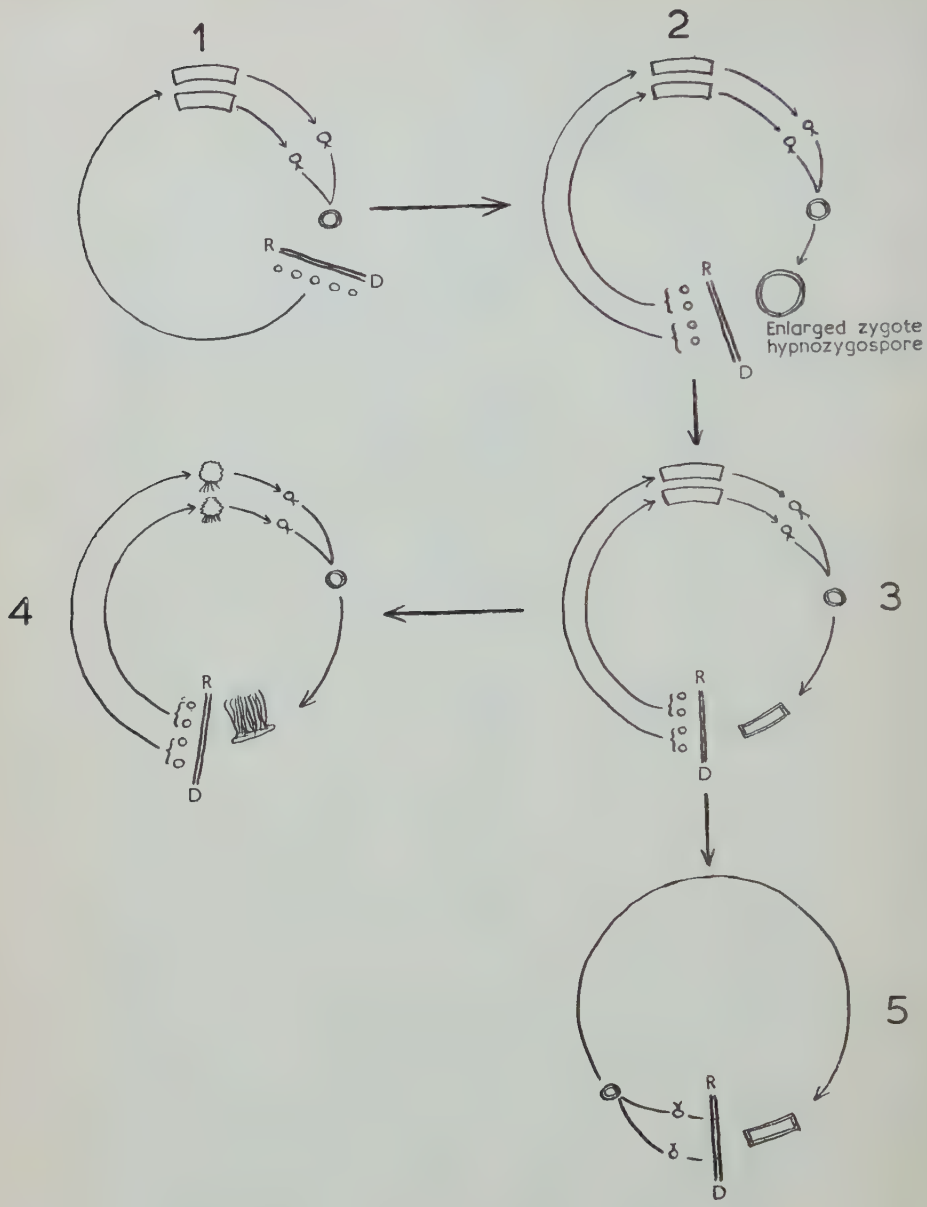


Fig. 1.

1. Monobiontic monogenic haplont.
2. Monobiontic digenic diplohaplont.
3. Dibiontic digenic isomorphic diplohaplont.
4. Dibiontic digenic heteromorphic diplohaplont.
5. Monobiontic monogenic diplont.

Double lines denote diploid phases.

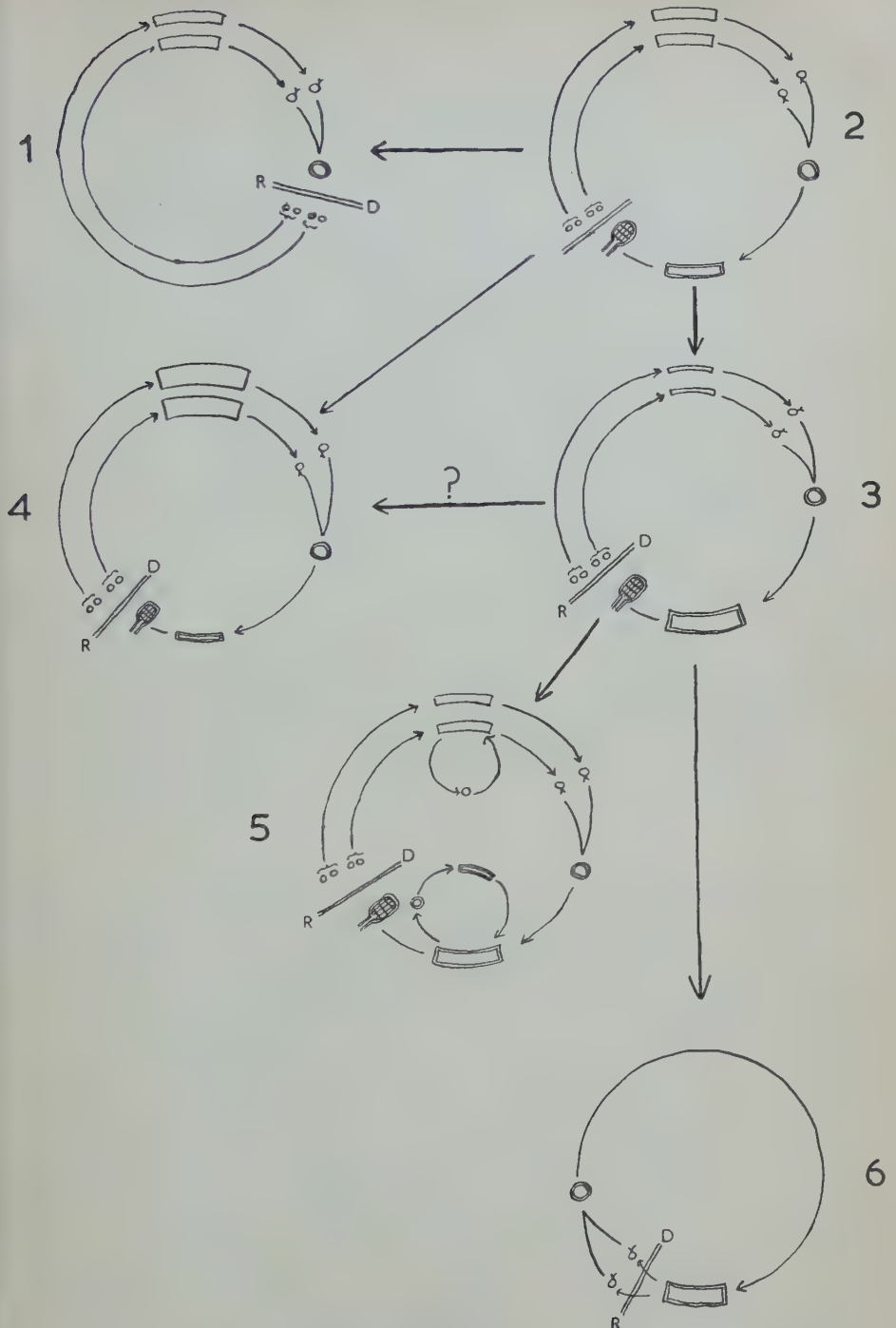


FIG. 2.

1. Monobiontic monogenic haplont. 2. Dibiontic digenic isomorphic diplohaplont.
3. Dibiontic digenic heteromorphic diplohaplont (sporophyte dominant).
4. Dibiontic digenic heteromorphic diplohaplont (gametophyte dominant).
5. Dibiontic trigenic dimorphic diplohaplont. 6. Monobiontic monogenic diplont.

Double lines denote diploid phases.

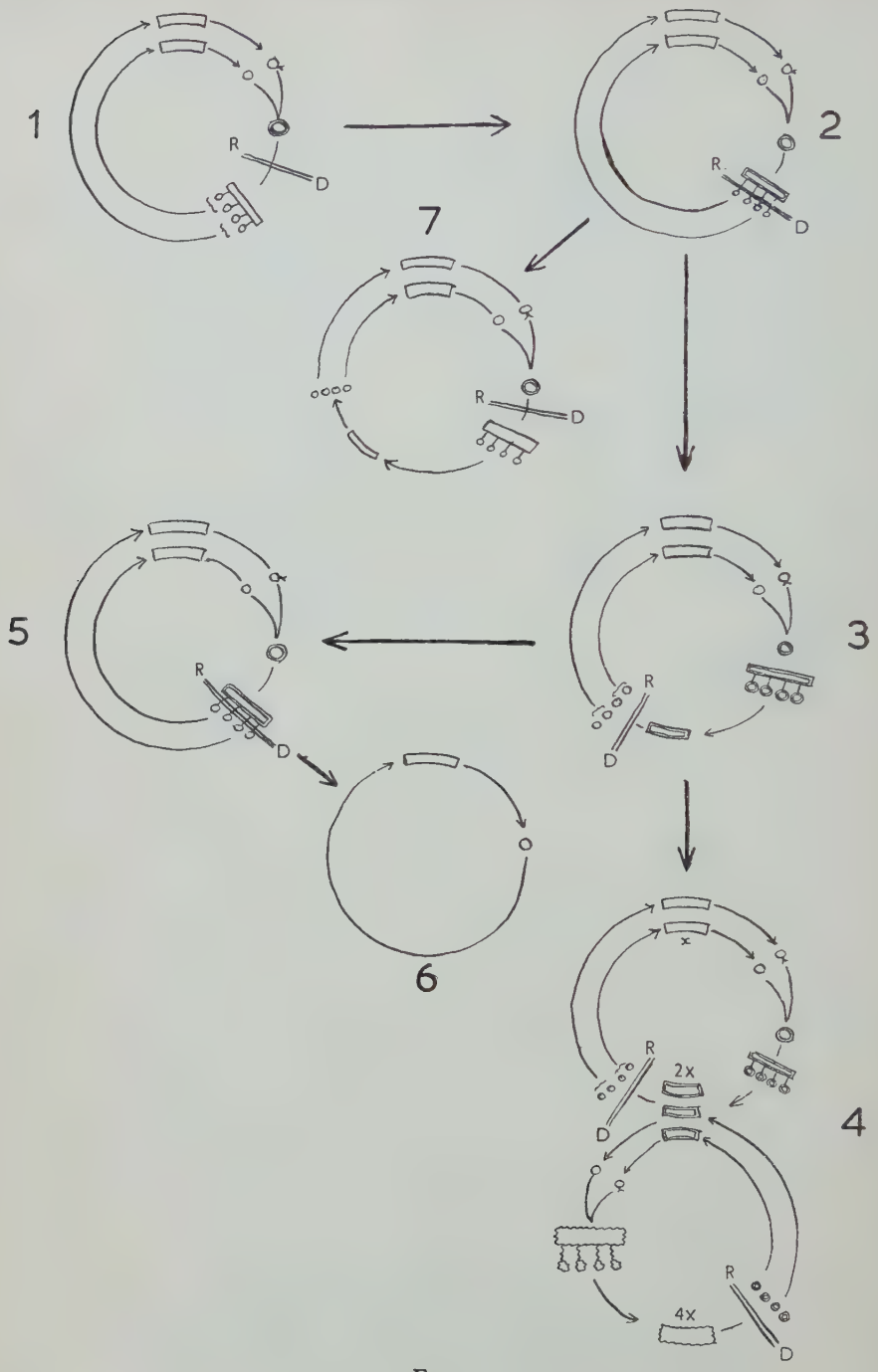


FIG. 3.

1. Monobiontic digenic haplont.
2. Monobiontic digenic diplohaplont.
3. Dibiontic trigenic dimorphic diplohaplont.
4. Polybiont.
5. Monobiontic digenic diplohaplont.
6. Ameiotic monobiontic monogenic (?) haplont.
7. Dibiontic trigenic trimorphic haplont. (In view of Magne's work this may have evolved from 1.)

Double lines denote diploid phases; wavy lines denote polyploid phases.

regarded as more primitive than the latter. Further development could legitimately give rise to the dibiontic, trigenic, dimorphic diplohaplont type as seen in some Dictyosiphonales and Chordariales. Reduction, on the other hand, leads to the Fucalean life-cycle (monobiontic, monogenic diplont; Fig. 2).

In the Rhodophyceae the primitive life history appears to us to be the monobiontic, digenic haplont type. This, with a gradual delay in the reduction division, would lead to the monobiontic, digenic, diplohaplont type and, with yet further delay, to the widespread dibiontic, trigenic, dimorphic diplohaplont. Recent work by Magne (1961) has shown that in *Nemalion helminthoides* meiosis does not occur at zygote germination so that the carpospores are diploid. On germination the carpospores give rise to little diploid plantlets, which do not give rise to new gametophytes. The plantlet is probably a tetrasporophyte and may or may not grow to a form similar to the gametophyte, but this is at present not known. It would seem unlikely that it does, because such plants would surely have been recorded. If the tetrasporophyte is a dwarf plant we have here an example of a dibiontic, trigenic, trimorphic diplohaplont. Magne (1960) has also shown that *Bonnemaisonia asparagoides* has the same kind of life history, the tetrasporophyte (*Hymenoclonium serpens* stage) being diploid. This work and any similar work may mean that the scheme in Fig. 3 will require reconsideration. Reduction, as seen in *Gymnogongrus griffithsiae* and *G. platyphyllus*, brings back again the monobiontic, digenic diplohaplont which in *Ahnfeldtia* reaches the condition where it must be described as an ameiotic, monobiontic, monogenic haplont. The polyploid series would seem to have evolved from the central group as an independent line (Fig. 3).

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